Marine yeast *Candida* MCCF 101 as feed supplement in aquaculture: nutritional quality, optimization of large scale production and evaluation of its protective effect on Koi carp from *Aeromonas* infection

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In partial fulfillment of the requirements for the degree of

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under the Faculty of Environmental Studies

by

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Ph.D. Thesis under the Faculty of Environmental Studies

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Certificate

This is to certify that research work presented in the thesis entitled **Marine yeast *Candida* MCCF 101 as feed supplement in aquaculture: nutritional quality, optimization of large scale production and evaluation of its protective effect on Koi carp from *Aeromonas* infection** is based on the original work done by Ms. Sunitha Poulose under my guidance at National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Declaration

I hereby do declare that the work presented in this thesis entitled **Marine yeast *Candida* MCCF 101 as feed supplement in aquaculture: nutritional quality, optimization of large scale production and evaluation of its protective effect on Koi carp from *Aeromonas* infection** is based on the original work done by me under the guidance of Dr. I. S. Bright Singh, Professor in Microbiology, School of Environmental Studies and Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Dedicated to God Almighty

who has been

my eternal rock and source of refuge

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Chapter 1

INTRODUCTION

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1.1 Aquaculture

Aquaculture is a rapidly growing industry recognized as a viable and profitable enterprise world over (Tacon, 1999). This has made its greatest advancements during the latter part of the 20th century (Lovell, 1998). The rapid increase in the global population directly caused increased consumption of fish globally creating a spurt in aquaculture promotional activities (Starkey, 1994). Fish and fish-based products are in very high demand because of their nutritional quality and health benefits (FAO-FD, 2006). Over the past 40 years, per capita consumption of fish has increased from 11 lbs to 16 lbs; some experts predicted that the per capita consumption of fish and shellfish would reach 25 lbs by the year 2025 (Parker, 2012). In this context, aquaculture is identified as the only viable alternative for any further increase in fish production to meet the ever increasing demand of fish and fish products.

1.2 Aquaculture nutrition

In aquaculture nutrition it becomes absolutely essential to supply feed to meet the nutritional requirements for enhanced growth and productivity (De Silva, 1993). This is because naturally available aquatic food may make a relatively small contribution to the total protein and energy requirements of the cultured fish, and this deficiency is made up with supplementary feeds. Therefore, inevitably, increase in fish biomass in culture systems become increasingly depended on the supplementary feed for all nutrients (Lovell, 1998). Feed supplements are expected to provide proteins, lipids, carbohydrates and energy as well as vitamins and micro and macro nutrients to enable fish to remain free from stress and diseases (Nakagawa *et al*., 2010). In this context microbes as feed supplements produced through biotechnological processes have been actively investigated as alternative or unconventional feed supplements for aquaculture and aquaculture systems (Banerjee *et al*., 2000).

Fish requires nutrients obviously for growth, reproduction and other normal physiological functions. Interest in fish and shellfish nutrition has been increasing markedly over the past two decades, largely due to the global increase in aquaculture production (Lall, 2000) and the awareness of the farming community on the requirement of balanced supplementary feeding for sustainability in the production. It has to be pointed out that majority research on finfish nutrition has been concentrated on to satisfy the requirements of carnivorous fish, however, adequate importance was given on omnivorous fishes as well. It has to be realized that increased understanding of the nutritional requirements of cultivable species of fish and shrimp coupled with improvements in feed manufacturing technology

and feeding techniques have been central to the expansion of modern aquaculture (Goddard, 1996). Feed cost represents about 50% of the variable operating cost in fish farming over the last 10 years, and the emphasis of research on nutrition and feeding has been on reducing the feed cost. The present aquaculture industry focuses much upon the relationship and importance of feeds and feeding with productivity and sustainability (De Silva and Anderson, 1995).

1.3 Nutritional requirements

Proper nutrition is one of the most important factors influencing the ability of cultured organisms to attain the genetic potential of growth, reproduction and longevity. Nutrition, immunological response and disease resistance in fish and other aquatic animals are important areas of research, which are in their infancy. The dietary requirements of fish and their sources (Trichet, 2010) are different from those of terrestrial animals and it is of paramount importance that they are processed properly in such a way that the essential nutrients are not lost during the process and their nutritional composition is sufficiently adequate for fish health. The essential nutrients are those for which fish or shrimp have an absolute dietary requirement and which they are unable to synthesize from other dietary ingredients. Lack of or insufficient level of these essential nutrients in diets may lead to stunted growth, deformity or the fish may be prone to diseases (Goddard, 1996). Subjects such as requirements and availability of the essential nutrients and their role in metabolism turn out to be the central themes of research in aquaculture nutrition. .

Nutrients are classified as macronutrients (protein, carbohydrates, lipid and energy) and as micronutrients (essential amino acids, fatty acids, vitamins and minerals). Of these nutrients, some are used for building up and maintenance of body structure and others supply energy. Proteins, lipids, carbohydrates are the nutrients that furnish the required energy for maintenance, activity, moulting, growth and reproduction. The non energy nutrients are the vitamins and minerals, which have very important and diverse physiological and biochemical functions and are extremely important in the attainment of nutritional efficacy of a feed (Ninawe and Khedkar, 2009). This realization of nutrient requirement of a cultured species, their source and role in metabolism are the essential factors for successful aquaculture.

1.3.1 Requirement of Energy

The energy requirement of fish and other cultured organisms is generally satisfied through protein, carbohydrate and lipids and releases during their metabolic oxidation. It is essential that it contributes to the utilization of all nutrients in a diet which is measured physically as calories of heat and physiologically as change in body weight, oxygen consumption and metabolic activity. Fish requires dietary sources of energy for growth, reproduction and health. This energy is spent for basal metabolism such as respiration, transport of ions and metabolites, body constituent turn over, circulation, voluntary or resting activity etc. Since all forms of energy are convertible to heat energy, the energy content of feed is conventionally expressed in units of heats (Calories). Diets formulated with excess energy may also promote excessive lipid deposition (Shearer, 1994) and reduced feed intake (Marais and Kissil, 1979).

1.3.2 Requirement of protein in fish

Protein is the dietary macronutrient whose requirement is for maintenance, growth, reproduction and for the replacement of depleted tissues and greatly affects weight gain. Among the fish feed components protein represents the highest fish feed cost and most expensive component in fish and prawn feeds (Guillaume, 1997; Meyer and Fracalossi, 2004). Optimum dietary protein level required for maximum growth in cultured fish and prawn is substantially higher than that of terrestrial animals.

Protein level in successful fish feeds range from 20-60% to accommodate differences in the physiological needs of different fish species (Pillay and Dill, 1979). Protein requirement for fingerlings of omnivorous species, such as channel catfish 28–32% (Robinson *et al*., 2000), Nile tilapia 30–36% (Shiau, 2002), Pacu 32-40% (Bicudo *et al*., 2010) common carp (Takeuchi *et al*., 1979; NRC (Anon, 1993) and flatfish species *Senegalese sole* 31- 35% (Rema *et al*., 2008). The gross protein requirements are highest in fry and it decreases as fish size increases. In the case of juveniles and adults of most cultured crustaceans protein requirement is in the range of 30-50% of their dry diet weight (Pillay, 1990). Protein requirements of shrimps present quite large variations from one species to another like *Metapenaeus macleayi* appears to be the one which requires the lowest (27%) protein in the diet followed by *Litopenaeus setiferus* having 28-32% requirement and *Marsupenaeus japonicas* which requires 40-57% (Takeuchi and Murakami, 2007).

Improper dietary protein to energy leads to increased fish production cost and deterioration of water quality resulting from wasted feed. The major symptom of protein deficiency or amino acid imbalance in fish is

reduced growth rate. High levels of protein and protein energy in the diet have resulted in depressed growth rate of catfish (Prather and Lovell, 1972).

1.3.3 Amino acid requirement

Nutritional research has demonstrated that dietary protein in general, and amino acids in particular, play fundamental roles in the overall activity of the immune system of fish (Gatlin, 2002; Li *et al*., 2009a). The various amino acids derived from proteins and deposited in fish tissue are grouped as essential and non essential. Essential amino acids such as arginine, methionine, valine, threonine, isoleucine, leucine, lysine, histidine, phenylalanine, and tryptophan (Takeuchi and Murakami, 2007) are indispensable in fish, crustaceans and molluscs feeds (Mambrini, 1995). Dispensable amino acids tested are alanine, aspartic acid, cysteine, glutamic acid, glycine, proline , serine, and tyrosine. Glutamine is crucial to the immune response and also increases weight gain in fish (Wu *et al*., 1996; Tannuri *et al*., 2000; Li *et al*., 2009a). Arginine plays important roles in the modulation of the immune response and enhances immune function during immunological challenges (Field *et al*., 2000; Calder and Yaqoob, 2004; Jobgen *et al*., 2006).

Dietary essential amino acid (EAA) requirement pattern in fish feeds has been determined for salmonids, catfish, eels, yellow perch and sea bream (Kaushik, 1998; Ninawe and Khedkar, 2009; Hart *et al*., 2010). Arginine was found to be indispensible amino acids for salmon (Klein and Halver, 1970), Channel Catfish (Robinson, 1981), Hybrid Striped Bass (Griffin *et al*., 1994), Rainbow trout (Kaushik *et al*., 1988) which was higher than that required for mammals (Pillay and Dill, 1979). Dietary

lysine requirement has been established for several species of fish, including Juvenile Hybrid Striped Bass, channel catfish, chinook salmon, chum salmon, Atlantic salmon, common carp, Japanese eel, rainbow trout, gilthead sea bream, several species of tilapia and red drum (Brown *et al*., 1988; Wilson, 1989; Griffin *et al*., 1992; Berge *et al*., 1998). These indispensable amino acids in proper proportion are the determinants for success or failure of any aquaculture industry.

Optimal dietary amino acid profile depends on the amino acid requirement of an animal based upon factors such as fish size, temperature, genetics, feeding rate, energy requirement, protein synthesis and other dietary factors (Ronnestad and Fyhn, 1993). Excessive amount of certain amino acids in the diet can lead to adverse reactions ranging in severity from reduced growth and feed intake to the occurrence of pathological lesions and death (Robinson *et al*., 1981). However, a single source of the protein may not supply all necessary amino acids and therefore it is recommended to have more such sources mixed in proper proportion while preparing the feed.

Free AA (FAA) are important energy sources for larval fish, from the embryonic phase until mouth opening (Ronnestad *et al*., 1992a, b; 1994; Finn *et al*., 1995). The FAA released in to the digestive system are then absorbed through the walls of the gastro intestinal tract in to the blood stream (Ronnestad *et al*., 1999) where they are then resynthesized to in to new tissue proteins or catabolised for energy or fragmented for further tissue metabolism. Salmonids and young carp are able to utilize diet containing FAA for growth (Ninawe and Khedkar, 2009).

Deficiency of essential amino acids may lead to poor utilization of dietary protein, and may result in growth retardation, poor live weight gain, and low feed efficiency. In severe cases, amino acid deficiency lowers resistance to diseases and impairs the effectiveness of the immune response mechanism. Deficiency of specific amino acids may also elicit clinical signs of diseases. For example, experiments have shown that tryptophan deficient fish become scoliotic, showing characteristic curvature of the spine (Kloppel and Post, 1975) and methionine or sulphur amino acid deficiency cause cataracts (Poston *et al*., 1977).These amino acids are not only incorporated in to fish proteins but also used for the synthesis of other compounds.

1.3.4 Carbohydrate

Carbohydrates are the most abundant and relatively least expensive source of energy which reduces environmental impacts in aquaculture (Martino *et al*., 2005). Developments in nutritional physiology, aquaculture technology and economical constraints have triggered the use of cheaper feed ingredients with higher grade of carbohydrates coupled with attractive physical characteristics and efficient energy utilization by the fish (Krogdahl *et al*., 2005). The nutritional value of carbohydrates varies among fish as fresh water and warm water species are generally able to utilize higher levels of dietary carbohydrates than cold water and marine species (Lall, 2000).

Omnivorous and herbivorous species (carp, channel catfish, tilapia, mullets, and milkfish) are capable of utilising 40-45% carbohydrate in the form of gelatinized starch. But carnivorous fish species (murrels, trout, sea bass, sea bream, and eels) have poor ability to digest carbohydrate as

compared to omnivorous and herbivorous fishes (Ninawe and Khedkar, 2009). Crustaceans require disaccharides and polysaccharides than monosaccharides (Takeuchi, 2007). In prawns, carbohydrates are important as an energy source, used for chitin and non-essential fatty acid synthesis.

Successful fish feed formulations contain carbohydrates to the tune of 20% for cold water and 30% for warm water fishes (Pillay, 1990). Increased dietary carbohydrate levels impair digestibility in salmon, carp and Pacu juveniles (Hernandez *et al*., 1994; Hillestad *et al*., 2001; Abimorad and Carneiro, 2007). Effects of mannan-oligosaccharide (MOS) in the diet improves feed utilization, promote growth, survival and disease resistance of Nile tilapia (Samrongpan *et al*., 2008) and sea bass (Torrecillas *et al*., 2011). The presence of certain carbohydrates may also enhance the texture and palatability of formulated feeds. Diet containing chitin and its precursor glucosamine has been shown to improve growth and feed efficiency in prawns.

Excess amount of starch causes sekoke disease in carp, which is similar to diabetes mellitus in warm blooded animals (Yokote, 1970) and enlarged livers in trout (Philips and Brockway, 1956) and catfish (Simco and Cross, 1966).

1.3.5 Requirement of lipid

Dietary lipids are important sources of energy and fatty acids that are essential for normal growth and survival of fish. They are rich sources of dietary phospholipids and are vital as structural components of bio membranes. They serve as vehicles for absorption of fat soluble nutrients such as sterols and vitamins. It is important that these sterols along with

certain fatty acids, must be provided preformed in the diets as they happen to be essential nutrients for fishes.

Lipids in the nutrition of fishes have emerged as key research area in connection with feed formulation for aquaculture species. Lipids have a special role in the immune mechanism of animals as some fatty acids serve as precursors of leukotrienes, 20-carbon compounds produced by macrophages and neutrophils having immunostimulatory functions (Lovell, 1998). In a diet, the lipid level determines the energy level also. Lipid levels in larval diets are generally high, 18% in the compounded diet of sea bream larvae (Salhi *et al*., 1999) 29–37% in *Artemia* for seabream larvae (Koven *et al*., 1992) and *P. olivaceus* (Furuita *et al*., 1998). It has also been pointed out that diets with excessive lipid content will increase lipid deposition to the visceral cavity, liver and in muscle tissues of fishes (Izquierdo *et al*., 2000; Gawlicka *et al*., 2002; Nanton *et al*., 2007).

1.3.5.1 Phospholipids requirement of fish

Phospholipids in general includes all those lipids containing phosphorus, having a central role in the development of structures of cell membrane bilayers, determining the hydrophobic and hydrophilic properties of the membrane surfaces as they do in mammals. It can serve as a source of energy in fish under certain circumstances, such as during embryonic and early larval development (Tocher, 2008). Intact phospholipids are required for optimal growth, survival, prevention of skeletal deformities, enhanced ovarian development and reproduction and possibly impart stress resistance in larval and early juvenile fish and help attain normal growth, moulting, metamorphosis and maturation in crustaceans (Xu *et al*., 1994;

Coutteau *et al*., 1997; Geurden *et al*., 1997; 1998; Weirich and Reigh, 2001; Wouters *et al*., 2001a, b; Cahu *et al*., 2003; Wu *et al*., 2010).

1.3.5.2 Fatty acids

Fatty acids are the main nutritionally active component of dietary lipids. The lipid component of the diet must provide an adequate amount of essential fatty acids for growth as well as for required dietary fuel (Bell *et al*., 2003). Therefore, sufficient amounts of essential fatty acids (ω-3 fatty acids or longer chain members of these series) must be included in the dietary lipids. Function of the essential fatty acids is to serve as a precursor to metabolic eicosanoids (20-carbon compounds). A few of these fatty acids are essential in the diet of fish and crustaceans, as they are not biosynthesised by these animals.

Essential fatty acids such as poly unsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA) are essential for normal growth, moulting and maturation of shrimp and enhance ovarian development and reproduction of various crustaceans (Xu *et al*., 1994; Sargent *et al*., 1999; Wouters *et al*., 2001a, b). PUFA are divided in to three families such as oleic acid, linoleic acid and linolenic acid where HUFA are as arachidonic, eicosapentaenoic and docasahexaenoic acid (Sargent *et al*., 1997). Essential fatty acid requirements have been demonstrated for several species of fish by the supply of 18:3n-3, 18:2n-6 fatty acids and 20:5n-3 and 22:6n-6 (National Research Council) (Anon, 1993) which appears to satisfy the requirement for maximum growth and efficient diet utilization (Lee and Sinnhuber, 1972).

Fatty acid deficiency signs reported for various fishes are elevated muscle water, loss of pigmentation, fin erosion, cardiac myopathy and

increased susceptibility to bacterial infection, reduced reproductive performance and shock syndrome (Castell *et al*., 1972). Essential fatty acid deficiency in rainbow trout reduces the invitro killing of bacteria by macrophages and reduces antibody production (Kiron *et al*., 1995).

1.3.6 Vitamins

Vitamins are essential growth factors to be essentially incorporated in fish and shrimp diets, for normal growth, reproduction and health. Most of these vitamins are required by animals in trace amounts, since they are either not synthesized within the animal's body or synthesized at too slow a rate to meet the animal's requirement. Vitamins can be divided in to two groups, the water soluble vitamins such as B and C group used rapidly after absorption, and the fat soluble vitamins such as A, D, E and K absorbed from the digestive tract in association with fat molecules and stored in fat reserves within the body. Four vitamins, such as vitamin A (Thompson *et al*., 1995; Hernandez *et al*., 2007; Yang *et al*., 2008), vitamin C (Hardie *et al*., 1991; Ai *et al*., 2004; Lin and Shiau, 2005a), vitamin E (Ortuno *et al*., 2000; Clerton *et al*., 2001; Lin and Shiau, 2005b) and vitamin B6 (Albrektsen *et al*., 1995; Feng *et al*., 2010) have been demonstrated to affect immune responses of fishes. The water soluble vitamin biotin is one of the most important and expensive vitamins added to aqua feeds (Li *et al*., 2010a; Yossa *et al*., 2011). It has been pointed out that B group vitamin riboflavin can improve growth, feed utilization, body composition and intestinal enzyme activities of juvenile Jian carp (Li *et al*., 2010b). Lin *et al*. (2011) demonstrated the requirement of folic acid and its effect on non specific immune response on grouper, *Epinephelus malabaricus*. The dietary ascorbic acid influences growth and morphogenesis in the larval

development of fish European sea bass (Darias *et al*., 2011). Fish store large quantities of vitamin D in their liver and fat tissues and do not synthesize it and are fully dependent on dietary sources to meet their requirement. Lock *et al*. (2010) reported the significance of vitamin D for mineral status, growth and development of fishes (Darias *et al*., 2011). They are generally required in relatively small amounts in the diet (Chen and Chang, 1994) and if not present in adequate amounts it may result in nutrition related disease, poor growth or increased susceptibility to infections. Shrimps such as *Penaeus monodon, Penaeus vannamei and P chinensis*, required dietary vitamin A for optimal growth (He *et al*., 1992; Chen and Li 1994b; Shiau and Chen, 2000) Excess amount of fat soluble vitamins may accumulate in the body causing vitaminosis or vitamine poisoning. Deficiency symptoms are discoloration, lack of coordination, nervousness, haemorrhage, lesion, fatty liver, and increased susceptibility to bacterial infections.

1.3.7 Minerals

Trace minerals are an essential component of fish nutrition which lags in inquiry when compared to research devoted to other nutrients. Minerals or inorganic elements are needed to animals to maintain many of their various biological functions such as the formation of skeletal tissue, respiration, digestion and osmoregulation. The essential minerals and trace elements are generally classified as either macro or micro ingredients depending on their concentrations within the body of fish or shrimp. Major minerals required in large quantities include calcium, phosphorus (Luo *et al*., 2010), sodium, potassium, chlorides, magnesium and sulphur (Rodehutscord, 1996). Trace minerals are those required in trace amount

namely iron, manganese, zinc, copper (Tan *et al*., 2011), iodine, cobalt, selenium, molybdenum, fluorine, aluminium, nickel, vanadium, silicon, tin, cadmium (Tan *et al*., 2010) and chromium. Zinc (Zn) is an essential trace element, required by fish for many important biochemical processes including growth, protein metabolism, antioxidant responses, energy production, gene regulation, and maintaining the health of cell membranes and bones (Lall, 1989; Watanabe *et al*., 1997; Yamaguchi, 1998; Luo *et al*., 2011). Most waters contain ionisable mineral compounds especially calcium which is available to fish directly through gills and skin. Calcium and phosphorous are closely related in metabolism especially in bone formation and the maintenance of acid base equilibrium. Magnesium plays a vital role as enzyme cofactors and as an important structural component of cell membrane. Dietary zinc (Zn) and selenium (Se) are both essential elements for metabolic processes and can be supplemented to meet dietary requirements for fish (Rider *et al*., 2010). The supplement of minerals should be considered for the maintenance of good growth and health. Symptoms of mineral deficiency in fishes results in a form of loss of appetite, deformed backs (Iordosis), poor growth, sluggishness, microcystic and hypochromic anaemia.

1.4 Non nutrient and non toxic dietary components

There are certain constituents of diets which have no apparent nutritive value and are not known to be toxic. These are fibre and ash. Fibre is not a specific chemical compound and all plant contains a certain amount of fibre. These certain amount of fibre in feeds permits better binding and moderate the passage of food through the alimentary canal. Fibre is indigestible it adds to the fecal waste and increase the biological oxygen

demand (BOD) in the culture system. Ash is a heterogeneous group of material, including the non combustible inorganic components of feed stuff and diets. In practical diets the ash content is not desirable to exceed 12%.The ash content affects the digestibility of diets, and would indirectly influence the culture.

1.5 Trends in feed development

The rapid expansions of aquaculture, along with improvements in fish culture techniques have increased the demand for fish feeds. The requirement of intensive aquaculture for nutritionally complete feeds or feed ingredients have stimulated considerable research and developmental activities in the field of fish nutrition and feed technology (New and Csavas, 1995; Goddard, 1996; Chandra *et al*., 2010). Apart from replacing fish meal with alternative feed source, there are other developments that could be expected in the aquaculture feed industry (Oliva-Teles and Gonçalves, 2001). The envisaged developments involved in aquaculture are that the diet should be formulated with an emphasis on minimizing environmental degradation, enhancing the quality of the produce and larval survival and growth. Other characteristics of feed include the production of acceptable fish, flesh quality, providing sufficient palatability and a positive effect on fish growth and health (Gatlin *et al*., 2007). These advances are particularly aimed at aqua feed for high value, intensively cultured species.

Meyers (1994) stated that significant growth has occurred in the number of feed manufacturing facilities worldwide as well as organizations involved in supplying feed ingredients or additives, live feed, vaccines, antibiotics, disease diagnostics, and other products that may be required for

successful aquaculture. Advances in diet development and nutrition will include use of alternative proteins in reduction of dietary fish meal and improvement in start-feeding diets for early stages of growth. Significant advances can be anticipated in analysis of nutritional requirements of additional finfish and shellfish species suitable for commercial culture. Such feed advancements have resulted in increased survival, higher growth and greater resistance to stress. This technology will become more prevalent in the predictable future (De Silva and Anderson, 1995).

1.6 Feed formulation

Diet formulation is the process in which the appropriate feed ingredients are selected and blended to produce a diet with the required quantity of essential nutrients and provide better health conditions. Feed is an important tool to prevent disease outbreaks and to improve production efficiency (Signor *et al*., 2010). By selecting various ingredients in the correct quantity and proportion a compounded diet, nutritionally balanced, pelletable and palatable is brought out which could be easily stored (Glencross *et al*., 2007).

The primary objective of feed formulation is to supply the species under culture an acceptable diet that meets its nutritional requirements at different stages of its life. In this process it is advised to use locally available feed ingredients considering the digestive capacity of the organisms for optimal animal production at minimum cost (Mazid *et al*., 1997; Árnason *et al*., 2009). The basic composition of feed formulation need the main information regarding the levels of crude protein, energy, specific amino acids, lipid, crude fibre and ash required in adequate

quantities and proportions (Lupatsch and Kissil, 2005; Irvin *et al*., 2010; Poppi *et al*., 2011). The energy level may be in terms of metabolizable energy (ME) or digestible energy (DE) (Allan *et al*., 2000).The type of culture system also has a determining role in feed formulation.

Many factors need to be considered in order of formulating a feed which will provide optimal growth performance of the cultured organisms while being it cost effective (Bautista *et al*., 2011). These factors are:

- Nutrient requirements: - It is of vital importance that a formulated feed meet the nutritional requirements of the cultured organisms.
- Composition of ingredients: - Knowledge of the nutrient composition and available energy of dietary ingredients is essential for their selection for use in diet formulation.
- Digestibility and nutrient availability: - If feed formulation has to be performed correctly, knowledge on the digestibility of the ingredients is essential.
- Other dietary components: - Certain ingredients are added to the diets for physiological or economic reason, which include binders, antioxidants etc.
- Dietary interaction: - micronutrient and macronutrient interaction are influenced by a number of factors including diet composition, diet processing, species and age of cultured organisms and environmental factors.

The important functions of formulated diet and preparations are (1) Provide the animals with their energy requirements as well as micro and macro nutrients (2) Supplement the natural food sources as in semi intensive culture which do not necessarily have to provide all the essential nutrient requirements (Abramo, 2002; Kader *et al*., 2010; Nakagawa, 2010). Nutritional deficiencies are encountered when the ingredients in the feed are not properly constituted or when it is fed in a stressful environment. An advanced method of fixing up ingredients is to compile their numerical coefficients to enable a computer to select them on the basis of chemical and physical properties which will optimize fish production at low feed cost (Allan *et al*., 2000). Properly formulated feed is an essential requirement for successful aquaculture (Meyers, 1994).

1.7 Forms of feed

Feeds supplied to aquatic organisms can be divided in to two major groups such as natural feed produced by fertilization within the culture system and supplementation of prepared feed. Natural feed is mainly fulfilled by fertilizing fish ponds with organic manure, inorganic fertilizers or combination of both. Prepared feed to the culture system includes live as well as artificial feed. The production of live food organisms for feeding fish and shrimp larvae is a major cost in hatchery operations; consequently artificial diets have been extensively researched upon as potential replacements.

1.7.1 Live feed

In nature fishes exist on live feed consisting of plants and animals available in their ecosystem. Fish larvae, especially, are unable to digest

the compounded diet because of insufficient digestive enzymes, but live feeds provide exogenous enzymes which are necessary for early stages of larvae to digest the food (Blaxter *et al*., 1983; Cataldi *et al*., 1987; Munilla-Moran *et al*., 1990; Kjorsvik *et al*., 1991; Hamlin *et al*., 2000; Kolkovski, 2001; Cahu and Infante, 2001; Lim *et al*., 2003). The initial sources of food for many larval organisms are phytoplankton. After a certain period of time, the larvae of most species except molluscs can be fed exclusively on zooplankton or other animal species or a combination of plant and animal matter available in nature or cultivated specially. In nursery and grow-out ponds, algal culture will be more convenient and they are generally produced as a result of fertilization and the availability of sunlight. Larvae of many species of fish and crustaceans grow well on live feed organisms, especially on zooplanktonic organisms such as brine shrimp or artemia, rotifers and copepods (Rosenlund *et al*., 1997; Abramo, 2002; García-Ortega *et al*., 2002; Langdon, 2003; Rajkumar and Kumaraguru, 2006; Mitra *et al*., 2007).

i) Algal culture

In recent years there is greater interest in intensive form of algal culture for generating live feed organisms for aquaculture especially in finfish and crustacean culture. Algal cultures are used mainly to feed the larval forms (Nakagawa, 2010) which are rich sources of micronutrients apart from protein, lipids and carbohydrates which influence growth and immune system. (Trichet, 2010; Dang *et al*., 2011). Several species of micro algae such as *Chaetoceros, Thalassiosira, Tetraselmis, Isochrysis, and Nannochloropsis* etc. are cultured to feed the larval stages in hatcheries (Duerr *et al*., 1998). Besides, mass cultivation of algae in ponds is also been carried out by fertilization which forms food for the cultured stock.

ii) Brine shrimp

Brine shrimp, *Artemia salina,* has been used as live feed organism for decades and receives considerable attention in recent years, especially in crustacean hatcheries (Koven, 2001; Abramo, 2002). *Artemia nauplii* are a standard larval diet for the fry production of marine as well as fresh water finfish and shellfishes (Van Stappen, 1996; Lim *et al*., 2003). Hatching of *Artemia* cysts and production of nauplii for feeding larvae in aquaculture is relatively easy. The *nauplii* have the highest calorific content and should be harvested and used at appropriate time.

iii) Rotifers

Rotifers are ideal starter feed for various ornamental fishes and it has greater nutritional value than *Artemia.* Rotifer *Brachionus plicatilis* is the most important live feed to larviculture of marine fish, and mass production of marine fish larvae is largely dependent on the availability of rotifers (Lim *et al*., 2003). *Brachionus calyciflorus* has better potential for application in larviculture of fresh water ornamental fish (Lim and Wong, 1997).

iv) Copepods

Copepods are considered to be "nutritionally superior live feeds'' for commercially important finfish and shellfish species and its nutritional composition matches with the nutritional requirements of the cultured stock (Stottrup *et al*., 1998; Shields *et al*., 1999; Finn *et al*., 2002; Hamre *et al*., 2002; Stottrup, 2003; Lee *et al*., 2005; Rajkumar and Kumaraguru, 2006; Vandermeeren *et al*., 2008). In the natural environment the vast majority of fish larvae show preference for copepods in the larval and adult

stages (McKinnon *et al*., 2003). A growing population of copepods is essential for larval survival, growth, digestion and metamorphosis (Hernandez-Molejon and Alvarez-Lajonchere, 2003).

However, with live feed it is not easy to ensure the required quality and quantity in order to provide the essential nutrients to the cultured stock in the required proportion. Therefore it becomes impossible to regulate natural food production synchronizing with the requirements through known fertilization or management practices. Because of these limitations, an aqua culturist has to depend on artificial feeds for intensified production.

1.7.2 Artificial feed

Artificial feeds should be scientifically formulated, optimally processed and judiciously supplied. Successful and sustainable aquaculture of finfish and crustaceans depend upon the provision of nutritionally adequate, environment friendly and economically viable artificial feeds (Fleming *et al*., 1996) which may be either a simple feed (e.g. rice bran, leaves etc.) or a formulated feed (e.g. wet, dry or pellet feeds). Natural feed supplies tend to vary in nutrient composition depending upon the factors such as stage of maturity of plant that constitute these supplies. Formulated feed can meet all the nutrient requirements for maximum growth in addition to adequate and easy application (Suckling *et al*., 2011).

1.7.2.1 Formulated or processed feeds

The main advantages of formulated and processed feeds are to utilize the knowledge of nutrient requirements, locally available feed ingredients and digestive capacity of the organism for the development of a nutritionally balanced mixture of feedstuffs which will be eaten in adequate

amounts to provide optimum production of the cultured organism at an acceptable cost (Bautista-Teruel *et al*., 2011).

To produce feeds with the desired physical characteristics, processing is required. Processed feeds are three types which are purified, semi purified and practical. Purified diets are comparatively expensive and are used only for research purpose e.g., synthetic amino acids, fatty acids and carbohydrates etc. Semi purified diets are commonly used as test diets in nutritional studies and it contains natural ingredients in as pure a form as is available e.g. casein oil, corn oil and fish oil etc. Practical diets are aimed at fulfilling the nutrition needs of the animal under culture at the minimum cost e.g. fish meal, oil seed meals and cereal grains etc.

1.7.2.1.1 *Feed ingredients*

The evaluation of feed ingredients is crucial to nutritional research and feed development for aqua culture species (Chandra *et al*., 2010). Fish feed ingredients are specially designed for the aquaculture industry to reduce incidence of disease and mortality and improve weight gain for shrimp, fish, and other aquatic species. They intend to use ingredients that are very high in energy to help reduce nitrogen waste production, increase feed efficiency and reduce the amount of feed fed to fish (Azevedo *et al*., 2004). Many of these ingredients are more complex than fish meal and require thorough evaluation in order to determine their nutritional value and appropriate use levels in prospective diets. Fish farmers have been looking for less expensive alternative sources of rich proteins and energy as fish feed ingredients. Proteinaceous ingredients are important constituents of the feeds and will usually comprise a significant part of the formulation. These

alternative protein sources have been derived from terrestrial animals, plant proteins and microbial proteins such as algae, bacteria, fungi.

i) Animal protein source

They constitute the most important and very expensive ingredients of aquaculture feeds but it contains unidentified growth factors. The potential of land animal protein ingredients as fish meal substitutes in diets for fish is very high (Wang *et al*., 2010). Animal protein such as blood, feather meal, poultry by products meal, fish meal, meat meal, raw fish, fish oils, fish silage, shrimp meal, and meal by products have considerable potential as feed ingredients in fish production systems (Dong *et al*., 1993; Fagbenro and Jauncey, 1995; Bureau *et al*., 2000; Cordova-Murueta and Garcıa-Carreno, 2002; Millamena, 2002; Nwanna *et al*., 2003; Amar *et al*., 2006; Hernãndez *et al*., 2010; Ye *et al*., 2011). The major problem with fish silage can result in lots of wastage and consequently result in increases in the biological oxygen demand (BOD) of the culture medium. High cost of the fish meal and shortage of supplies have led to the exploration of substitutes.

ii) Plant protein sources

Many plant ingredients have been evaluated as protein source in fish feeds (Carter and Hauler, 2000; Gatlin III, 2007; Nguyen and Davis, 2009; Nyina-Wamwiza *et al*., 2010) but their value gets reduced due to the presence of antinutritional factors such as protease inhibitors, non-digestible carbohydrates, lectins, saponins, phytates and possibly allergenic storage proteins (Rumsey *et al*., 1995; Hardy, 1996; Francis *et al*., 2001; Zhou *et al*., 2004). Most protein rich plant ingredients are characterised by having the majority of their phosphorus bound in phytate. This phytate is

limited in bioavailability to most fish because they lack the digestive enzyme phytase (Lall, 1991; Jackson *et al*., 1996; Riche and Brown, 1996). This problem is solved by the existing biotechnological innovations to remove the enzyme inhibitors and antinutritional factors by feed enzymes like phytases. In this context cereal proteins can also play a more significant role in formulated feeds in the future (Rodehutscord and Pfeffer, 1995; Schafer *et al*., 1995; Van Weerd, 1999; Papatryphon and Soares, 2001; Cao *et al*., 2007).

iii) Microbial protein source or Single Cell Protein (SCP)

Predictions of future protein shortage have spurred research on non agricultural methods of protein production. In more recent years advances in scientific knowledge regarding the physiology, nutrition and genetics of micro organisms have led to significant improvement in single cell protein production from a wide range of micro organism and raw materials. SCP is excellent source of nutrients such as well balanced amino acid profile, vitamins, minerals and also possess usable lipids and carbohydrates. They are high in protein contents and may also increase as replacement ingredients for fish meal in aquaculture feeds (Bob-Manuel and Alfred-Ockiya, 2011). Feed requirements directly affect fish growth and for the furtherance of growth, suitable supplementary feeds in sufficient quantity are acquired. These types of high protein products suitable for feeding to livestock and fish are now produced from single cell organisms. At a minimum these organisms require a carbon and energy sources, nitrogen sources and supplies of other nutrients for growth in a water environment. This type of feedstuffs is mainly derived from unicellular organisms such as algae, bacteria, fungi and yeast. Advantages of single cell protein include

the inherent high protein content, rapid increase in cells in short time and independence of climatic conditions for growth.

iv) Algae

Algal biomass has the potential to enhance the nutritional content of conventional feed preparations and to act as a feed additive to supply basic nutrients and pigments in fish culture in several ways (Brown, 2002; Becker, 2004; Zmora and Richmond, 2004; Zatkova *et al*., 2010; Dang *et al*., 2011). Algae are better source of SCP due to their ability to undergo photosynthesis by using CO_2 as carbon source and solar radiation as energy source. So, light intensity and temperature are the important factors for algal growth. Artificial illumination is too costly to use for producing algal SCP as an animal feed stuff. The various genera of algae employed for SCP are C*hlorella, Duanliella, Scenedemus* and *Spirulina* etc (Chi *et al*., 2006; Nakagawa, 2010). Algae are generally cultivated under non sterile, mixed, culture conditions in location where the environmental condition favour the dominance of the desired algal species over competing species. The outdoor cultivation of algae for SCP production involves a coupling of agricultural technology and industrial microbiology. They can be operated in an optima manner by controlling the CO_2 supply, fluid flow, and agitation of the systems. This contributes significantly to the practical production of algal SCP.

v) Bacteria

Many species of bacteria have been investigated for use in SCP production but some cannot be of use if they are pathogenic for plants, animals and human. The bacterial cells are known to have comparable

protein content than the other microorganisms such as algae and yeast (Kobayashi and Kurata, 1978). Nonetheless the quality of the bacterial protein with respect to amino acid composition is better due to higher amount of sulphur containing amino acids and lysine. Various genera of bacteria employed in the SCP production include *Bacillus, Cellulomonas, Pseudomonas, Rhodopseudomonas Palustris, Lactobacillus* and *Methylococcus, Rodovulum sulfidophilum* etc. (Getha *et al*., 1998; Banerjee *et al*., 2000; Gomez-gil *et al*., 2000; Kim and Lee 2000; Abraham *et al*., 2008). Maintaining sterile conditions during SCP production is very important because contaminating microorganisms grow very well in the culture medium. Limitations of bacterial SCP are its smaller size than yeast and also costly to harvest unless the cells can be flocculated to give higher solids prior to centrifugation. These have only brief history of use as either animal feed or human food.

vi) Fungi

The use of fungal SCP as a functional ingredient in foods is an especially interesting possibilities as an aquaculture feed ingredient (Rodriguez *et al*., 2004; Nitayavardhana and Khanal, 2011). Higher fungi such as mushrooms have been used as a staple food for centuries. The filamentous fungi used for SCP are *Aspergillus, Pencillium, Fusarium, Rhizopus, Ganoderma* and *Gladosporium* (Anupama and Ravindra, 2001). Their slow growth, lack of sulphur containing amino acids and proneness to bacterial contamination are the major disadvantages. A recent trend in SCP production is the exploration of fungal species for bioconversion of lignocellulosic wastes.

vii) Yeast

Yeast single cell protein has long history of human consumption and most commonly used in animal and feed formulation and dietary supplements. The risk of bacterial contamination is low and recovery is very easy. Nowadays different types of yeasts are widely used such as baker's yeasts, brewer's yeasts, nutritional brewer's yeasts, wine yeasts, distillers' yeasts, probiotic yeasts, yeast extract, and torula yeast. (Bekatorou *et al*., 2006). Industrial yeast is commonly used in aquaculture, either as live food organisms, or after processing, as a feed ingredient (Stones and Mills, 2004).Yeast by-products from the brewing industry are natural diet additives that have been shown to positively influence non-specific immune responses (Siwicki *et al*., 1994; Anderson *et al*., 1995) as well as growth (Rumsey *et al*., 1991b; Oliva-Teles and Goncalves, 2001; Ozorio *et al*., 2010) of some fish species and shrimps (Burgents *et al*., 2004). Yeasts are good sources of vitamins B, E, and D. Many species of yeasts used as sources of SCP are *Pichia, Candida, Saccharomyces, Kluyveromyces*, *Torulopsis*, *Hansenula*, *Koloechera* etc.

1.8 Yeast as a SCP

Yeasts are the principal organisms that have been used for the production of single cell protein. Today, yeasts are also used as alternative sources of high nutritional value proteins, enzymes and vitamins and have numerous applications in the health food industry as food additives, conditioners and flavouring agents for the production of microbiological media and extracts, as well as livestock feeds. Alternative protein sources, single cell proteins (SCP) such as yeasts have good potential, due to their high nitrogen content (Tacon and Jackson, 1985; Tacon, 1994; Anupama

and Ravindra, 2000).Yeast can supply the feed with the B –complex vitamins, minerals and other components which could stimulate the disease resistance of marine animals. Yeasts such as *Saccharomyces cerevisiae*, *Candida utilis, Candida tropicalis* and some species of genera *Hansenula, Pichia* and *Torulopsis* can be used for their single cell protein (Ravindra, 2000; Zhenming *et al*., 2006). Several studies have shown that yeasts can successfully replace part of dietary fish meal in different fish species (Mahnken *et al*., 1980; Rumsey *et al*., 1991a; Metailler and Huelvan, 1993; Takii *et al*., 1999; Oliva-Teles and Goncalves, 2001; Fournier *et al*., 2002; Olvera-Novoa *et al*., 2002). Studies conducted with fishes and shrimps have shown that yeast and yeast derivatives are effective growth enhancers and immunostimulants (Rumsey *et al*., 1991b; Siwicki *et al*., 1994; Noh *et al*., 1994; Scholz *et al*., 1999; Oliva-Teles and Goncalves, 2001; Ortuno *et al*., 2002; Suphantharika *et al*., 2003; Taoka *et al*., 2006; Abdel-Tawwab *et al*., 2008; Andrews *et al*., 2011). The oral administration or injection of the yeasts *Saccharomyces cerevisiae* or *Candida utilis* has been shown to increase both humoral (myeloperoxidase and antibody titer) and cellular (phagocytosis, respiratory burst and cytotoxicity) immune responses, and to increase or confer resistance against pathogenic bacteria in fishes (Chen and Ainsworth, 1992; Siwicki *et al*., 1994; Ortuno *et al*., 2002; Rodríguez *et al*., 2003).Yeasts contain various immunostimulating compounds such as β-glucans, nucleic acids, and oligosaccharides, and it has the capability to enhance the growth of various fish species (Gannam and Schrock, 2001; Oliva-Teles and Goncalves, 2001; Lara-Flores *et al*., 2003; Abdel-Tawwab *et al*., 2008; Abdel-Tawwab *et al*., 2010) and also its influence reported on growth performance and survival of juvenile prawn *Macrobrachium amazonicum* (Hisano *et al*.,2008) and the immunostimulant

properties (Ortuno *et al*., 2002; Rodríguez *et al*,. 2003; Esteban *et al*., 2004; Li and Gatlin 2005). The major components of yeast cell walls are β-1, 3 / 1, 6-glucans which are known as potent immunostimulants and has significant augmenting effects on the host defence systems of aquatic animals (Cabib *et al*., 1982; Sakai, 1999; Yadomae and Ohno, 1996; Gatlin III, 2002; Couso, 2003; Burgents *et al*., 2004: Selvaraj *et al*., 2005; Pais *et al*., 2008; Li *et al*., 2009b) for many years. Tiger shrimp immersed in yeast glucan solution (0.5 and lmg mL^{-1}) showed increased protection against *V. vulnificus* infection (Sung *et al*., 1994).Yeast culture feed supplement was assessed for its impact on disease resistance in the pacific white shrimp, *Litopenaeus vannamei* (Burgents *et al*., 2004). Yeast glucan also has adjuvant effects in aquaculture fish feed and the abilities to enhance the lysozyme activity complement activity and bacteria-killing activity of macrophages of marine animals and the production of superoxide dismutase by macrophages or hemocytes in some marine animals (Sakai, 1999; Dalmo and Bogwald, 2008).

Recent studies have shown that marine yeasts could produce bioactive substances with potential application in mariculture, food, pharmaceutical, cosmetic, chemical industries and environmental protection. Gama *et al*. (2001) and Zhenming *et al*. (2006) found that the marine yeasts were capable of adhering to the intestinal mucosa, thus providing potential applications as a probiotic supplement for human and animal health. Sukumaran *et al*. (2010) reported marine yeast glucans confer better protection than that of baker's yeast in *Penaeus monodon* against white spot syndrome virus infection. Brown *et al*. (1996) evaluated the possibility of using marine yeasts *Debaromyces hansenii* ACM 4784, *Dipodascus capitatus* ACM 4779, *Dipodascus* sp. ACM 4780 as feed for

bivalve culture. Sajeevan *et al*. (2009a) observed that marine yeast biomass is directly added to the larval rearing system as feed supplement for better protection and survival against microbial infection. However, all the marine yeast strains lacked the 20:5n-3 and 22:6n-3 fatty acids, making them unsuitable as a complete diet for larval raising (Brown *et al*., 1996)

Most *Candida* spp. has biological and biotechnological importance, and also potential applications in agriculture, fermentation industry and chemical industry. For example, *C. rugosa* and *C. antarctica* have been extensively used for commercial production of lipases, which are used in flavor industry, synthesis of lipophilic antioxidants, detergent industry, lipid hydrolysis, biosensors and clinical purposes (Hasan *et al*., 2006). *C.famata* (Stahmann *et al*., 2000) has been used for riboflavin biosynthesis on large scale. *Candida* sp. M15, *C.utilis* and *C. tropicalis* have been used for single cell protein production (Ravindra, 2000). *C. oleophila* can be used as a biocontrol agent against *Botrytis cinerea* and *Penicillium expansum* on harvested apples. Barnett *et al*. (2000) reported genus *Candida* isolated from marine environments, such as *C. aaseri, C. albicans, C.atlantica, C. haemulonii, C. intermedia, C.maris, C.maritima, C. norvegica, C. sake, C. torresii, C. tropicalis, and C. zeylanoides*. Most diets, at best, have served as supplements rather than complete substitutions (Kumlu and Jones, 1995). Sajeevan *et al*. (2006; 2009b) reported the efficacy of *Candida sake* S165 and its cell wall component β-glucan as immunostimulants in *Fenneropenaeus indicus.*

1.8.1 Nutritive value of yeast

The nutritional value of yeast depends on the composition of its carbohydrates, fats, proteins, amino acids, vitamins, nucleic acids and

minerals. The acceptability of a yeast as food or feed depends on its nutritional value and safety (Jay, 1996; Ravindra, 2000). Yeast contains lysine, thiamine, biotin, riboflavin, niacin, folic acid, choline etc but it is deficient in methionine. The yeast biomass has very high percentage of proteins. These proteins are easily digestible as compared to those from bacteria. Yeast protein contains all the essential amino acids. In some studies, supplementation of yeast-containing diets with limiting amino acids improves fish performance (Nose, 1974; Bergstrom, 1979; Spinelli *et al*., 1979; Murray and Marchant, 1986). One limitation to the use of high levels of yeast in fish diets is its relatively high non protein nitrogen content, in the form of nucleic acids. But dietary nucleotides have beneficial effects, as they positively affect the immune system, hepatic function, lipid metabolism, disease resistance, and growth (Carver and Walker, 1995; Cosgrove, 1998; Carver, 1999; Burrells *et al*., 2001; Low *et al*., 2003; Li *et al*., 2004). It is also a good source of vitamins especially vitamin B. The nutritional value such as amount of energy for metabolic process, protein content, nucleic acid level, amino acid level, carbohydrates, minerals, fatty acids, vitamins, trace elements etc. should be analysed before the product is used for food or as feed supplement. Some yeast, like *Candida* sp. and *Saccharomyces cerevisae*, are also believed to have immunostimulatory properties by virtue of their complex carbohydrate components and nucleic acid content (Anderson *et al*., 1995).The nutrient value of SCP may have a negative and positive impact. The rigid cell wall, high nucleic acid, allergies and gastrointestinal effect should be considered as a negative effect if it is used as human food. The high amount of protein with sufficient enzymes, minerals and vitamins bring positive effect on animal feed.

1.8.2 Production process of yeast

All possible conventional methods for the production of SCP should be thoroughly examined for their possible economic application. All the factors such as pH, temperature, humidity, oxygen requirement, carbon and nitrogen sources ought to be optimized thoroughly before using the production on a large scale. In the last decades, statistical experimental methods have been applied to media optimization for industrial purposes because of various advantages in their use (Srinivas *et al*., 1994; Carvalho *et al*., 1997). The application of statistical experimental design techniques in fermentation process development can result in improved product yields, reduced process variability, closer confirmation of the output response to nominal and target requirements and reduced development time and overall costs (Elibol, 2004). The optimization studies using response surface methodology (RSM) is an efficient experimental strategic tool to obtain optimum conditions for a multivariable system efficiently and this method have been successfully applied for optimization of fermentation media for various microorganisms (Montgomery, 1997; Medeiros *et al*., 2000; Rao *et al*., 2000; Adinarayana and Ellaiah, 2002; Balusu *et al*., 2005; Ratnam *et al*., 2005; Raoa *et al*., 2006; Li *et al*., 2008; Sheng *et al*., 2009).The central composite design (CCD) and response surface methodology (RSM) could be used to find out the relations between the variables and response, moreover, the optimum of every variable would be obtained by differential approximation. This technique has been used to study the optimization of physicochemical parameters and factors of many fermentation medium and process with various microorganisms (He and Tan, 2006; Gonen and Aksu, 2008; Lal *et al*., 2009). Production process development is carried out in three distinct steps, (i) Laboratory process

development using flasks, (ii) Scaling up using small to medium fermentors and (iii) Production scale fermentation experiments. Optimization of biomass productivity requires that the specific growth rate and biomass yield in the fed-batch process be as high as possible (Van Hoek *et al*., 1998).Yeast biomass can be produced very efficiently and economically because of their shorter generation time and use of inexpensive culture media. Konlani (1996) optimized the cell yield of *Candida krusei* and *Saccharomyces* sp. cultured in sorghum hydrolysate. Gonen and Aksu (2008) used response surface methodology (RSM) in the evaluation of growth and copper (II) bioaccumulation properties of *Candida utilis* in molasses medium. He and Tian (2006) used response surface methodology to optimize culture medium for production of lipase with *Candida* sp. 99-125.

1.8.3 Substrates used for yeast

Yeast requires a carbon and energy source, nitrogen source and supplies of other nutrient elements, including Phosphorus, Sulphur, Iron, Calcium, Magnesium, Manganese, Potassium and trace elements for growth in a water environment. The cost effective production of SCP is directly depended on the raw material and efficiency of the process of conversion of these raw materials in to described biomass. The choice of substrate is normally determined by its local availability as high transportation cost for carrying the substrate over a long distance is not recommended. Utilization of locally available waste material for SCP production is efficient for controlling the environmental pollution and is essential for cheaper production cost.

Many raw materials have been considered as substrates (carbon and energy sources) for SCP production. Yeast species are cultured on different

substrates mainly wastes from agricultural, forestry and food industry. These are sulphite waste liquor, sorghum hydrolysate , Jerusalem artichoke, prawn shells wastes, dairy wastes, whey, bagasse, wood pulp, wheat bran, paper mill waste, food processing waste, molasses and corn steep liquor, rice polishings etc. which provide carbon and nitrogen for growth (Bajpai and Bajpai, 1991; Nigam and Vogel, 1991; Konlani *et al*., 1996; Rishipal and Philip, 1998; Cristiani-Urbina *et al*., 2000; Lee and Kim, 2001; Moeini *et al*., 2004; Rajoka *et al*., 2004; Rivas *et al*., 2004; Bekatorou *et al*., 2006; Nasseri, 2011). Molasses is obtained mainly as a by-product of sugarcane and sugar beet refining. Beet molasses was used for SCP production by growing *Candida utilis* as supplement for human food. Ram horn hydrolysate used as carbon source for the single cell production of *Candida utilis*, which are significant waste products of the meat industry in Turkey (Kurbanoglu, 2001). Study showed that the new marine yeast *Cryptococcus aureus* G7a was suitable for single cell protein production from Jerusalem artichoke extract used as carbon source (Lingmei *et al*., 2007). A possible cheap production for single cell protein from yeasts Debaryomyces *hansenii, Kluyveromyces marxianus* and *Pichia stipitis* using non – detoxified breweries spent grains hemicellulosic hydrolyzate has been reported (Duarte *et al*., 2008).

1.8.4 Scale up of production of yeast in fermentors

Shaking bioreactors are very widely applied in academia and in bioindustry for screening and bioprocess development projects. Shake flask (250-500ml) cultures are a convenient method of growing microorganisms in submerged cultures under aerobic conditions created by shaking (Buchs, 2001). The information obtained from shake flasks is evaluated using small fermentors (1-12L). The information gained from these smaller fermentors

is used to determine the proper fermentation condition for the large (100-300L) and very large (1000L) fermentors, called scale up of the process. In microbial fermentation, variables which are of great relevance to the economic evaluation of such biotechnological processes are the cell yield on a particular substrate, specific growth rate coefficient, substrate consumption rate, specific substrate consumption rate, product yield based on substrate, specific product yield and rate of product formation (Tobajas and Garcia-Calvo, 1999). An appropriate low cost bioprocess technology for mass production has to be developed for commercial application. Marine yeast would be a better candidate as alternate for fish meal for large scale production according to their easy cultivation in the fermentor and high cell density. Rajoka *et al*. (2006) developed and optimized a fermentation process for the production of single cell protein by culturing *Candida utilis* on rice polishing in 14 and 50 L aerated fermentors. Cheese whey fermentation for the production of single cell protein using the yeast *Kluyveromyces fragilis* was developed by Ghaly *et al.* (2005). Large scale biomass production facilities using the proven technology of submerged fermentation due to better monitoring and ease of handling has been developed.

1.8.4.1 Submerged fermentation

Submerged fermentation is generally applicable for the manufacturing of cell products by propagation of microorganism and cell cultures in a fluid nutrient medium. In the case of submerged fermentation the biomass and the substrate are present in the submerged state in the liquid medium consisting of stirred or non stirred process and it confers upon many advantages like operational convenience, economy, reduced requirement of

space, greater reliability in inoculation and growth pattern, improved contamination control, and precise control of parameters (Sandhya *et al*., 2005; Agarwal *et al*., 2006; Mukherjee and Rai, 2011). Submerged fermentation is reported for production of biomass and from various microorganisms (Abadias *et al*., 2003; Kim *et al*., 2003). Submerged fermentations are mostly operated as batch, fed-batch and in continuous mode.

Batch process has received increasing attention in the second half of the twentieth century. In this case all ingredients used in the operation are fed to the processing vessel at the beginning of the operation and no addition and withdrawal of material takes place during this process. Under this condition the cell cultures pass through six phases of growth such as lag, acceleration, log, deceleration, stationary and death phases. It is important to monitor batch fermentations closely to ensure that the cells are harvested at the appropriate time. The best advantage of batch processing is the optimum levels of product recovery.

In fed batch mode the substrate and other nutrients are added in an incremental manner usually to prolong the log phase of the fermentation process. Controlled addition of nutrients in a fed batch operation allows for control of cell growth and thereby promotes production of secondary metabolites. Production of yeast is mostly by fed batch culture, where biomass and other nutrients are the desired products (Rodrigues *et al*., 1998; Lee and Kim, 2001). Mass production of *Rhodopseudomonas palustris* as diet for aquaculture by fed batch fermentation has been developed (Kim and Lee, 2000).

In a continuous culture operation nutrients essential for growth are continuously fed and a portion of the culture is continuously withdrawn. A continuous culture is usually preceded by a batch or fed batch culture. In the continuous processing, the rate of consumption of nutrients and those of the output chemicals are maintainable at optimal levels. Continuous process may increase the chance to contamination. Continuous culture is the ideal method for the production of microbial biomass (Ziino *et al*., 1999; Lee and Kim, 2001)

1.8.5 Downstream process

The efficient and economic fermentation of a product is not the end of a successful production process as the product must be recovered from the fermentation medium, processed and packaged with equal efficiency and economy. The down stream process determines 50% of the cost and has significant influence on product cost, purity and reactivity. Well designed downstream process determines how quickly a new product can be introduced in to the market place. Industrial yeast production generally involves such as propagation, involving a number of fermentation process, harvesting, centrifugation, packing and shipment. The packaging method varies among manufacturers and depends on the type of yeast product.

1.8.6 Advantages

Advantages of yeast SCP: Better public acceptance, lower content of nucleic acids, easier harvesting due to size and concentrations, better growth characteristics in substrate of low pH. Torula yeast especially *Candida utilis* grow rapidly. Capable of being formulated in to a variety of processed foods.

1.8.7 Application

The overall utilization of SCP falls into two categories: food and feed. The use of protein product in feed is related primarily to consideration of nutritional value and balance of ingredients. The high protein content, good storage properties in dry form, texture, blend odour and taste of SCP suggest real potential in food and feed markets.

i) SCP for human consumption

The level of utilization of yeast in food is certainly limited by the concentration of nucleic acids in the final product. Obtaining regulatory approval for the production of SCP for human consumption is even lengthier and more expensive process. A safety aspect that must be considered of all SCP products is nucleic acid content. The purine bases of nucleic acids are degraded in human body to uric acid. Increased level of this acid in blood and urine can cause gout or kidney stones. Gastrointestinal disturbances are common complaints in man following the consumption of algae and bacteria SCP

ii) SCP in animal feed

The animal feed market is more attractive for yeast SCP. The cells may be used directly as protein source in animal feed formulation and feed ingredients. In the case of animal feed or feed supplements, the dried cells may be used without further processing. Supplemental dietary yeast stimulated nutrient assimilation in ruminants and improved performance and carcass traits in swine (Newbold *et al*., 1996; Moreira *et al*. 1998; Huige, 2006). SCP can be used in animal feed without problems including, cattle, poultry, rabbits and lambs. Schwarz and Mertz (1959)

reported that extract of brewer's yeast could reverse the impaired tolerance to glucose load in yeast fed rats. Lower animals can degrade uric acid to the soluble compound allantoin and consequently the consumption of high level of nucleic acids does not present metabolic problems to these animals.

iii) SCP in aquaculture nutrition

Single Cell Protein (SCP) appears to be an attractive product in aquaculture feed market and it has seen the emergence of evermore sophisticated feed for fish cultivation (Tacon, 1994). SCP seems to be a potential source of protein and is playing a greater role in the evolution of aquaculture especially to fish and crustacean (Nayar *et al*., 1998; Ricci *et al*., 2003). The demand for SCP production increased worldwide in aquaculture industry for the production of shrimp, prawn, trout, salmon, carp and other finfish and shell fishes. Yeast SCP has been recognized to have potential replacement for fishmeal (Rumsey *et al*., 1991a; Rumsey *et al*., 1991b; Oliva-Teles and Gonc¸alves, 2001; Olvera-Novoa *et al*., 2002). Various species of yeast, and different yeast-containing products, have been fed to fish for an assortment of reasons. Yeast single cell proteins have been viewed as promising substitute for fish meal in fish diets (Li and Gatlin, 2003). These natural feed additives positively influenced the non-specific immune responses of many aquaculture species (Siwicki *et al*., 1994; Anderson *et al*., 1995; Thanardkit *et al*., 2002). The ingestion of yeast and fungal biomass appears to increase resistance to mycoses which often decimate fish farms. *Candida* sp, *Hansenula* sp, *Pichia* sp, and *Saccharomyces* sp have special importance as components in fish feeds (Ahmad and Abdel-Tawwab, 2011). Administration of yeast has been

recognised to have important effect in the functioning of immune system (Sakai, 1999).

1.8.8 Yeast SCP used as feed supplement

Advances in fish nutrition have progressed considerably with regard to growth performance. Supplementary feeding in semi-intensive aquaculture has to be considered in the perspective of the past and future trends of the industry (De Silva, 1993). A variety of feed supplements have been investigated with respect to their effects on the physiological condition and quality of cultured fish. According to World Research Institute, Chinese researchers have developed a protein supplement based on yeast that can substitute for more than half of the fish meal in aquaculture feed preparations. Brewer's yeast (*Saccharomyces cerevisiae*) has been used as a feed supplement for various fishes and it contains various immunostimulating compounds such as β-glucans, nucleic acids, mannan oligosaccharides and other cell wall components (Oliva-Teles and Goncalves, 2001; Li and Gatlin, 2003; 2005; Cuesta *et al*., 2007). Numerous studies have been focused on the effects of dietary inactive, dried and live yeasts culture supplement for growth performance, immunity and survival of fish and shrimp (Nell, 1985; Itami *et al*., 1994; Noh *et al*., 1994; Nell *et al*., 1996; Scholz *et al*., 1999; Lara-Flores *et al*., 2003; Stones and Mills, 2004; Barnes *et al*., 2006; Reyes-Becerril *et al*., 2008; Chiu *et al*., 2010; Tovar-Ramírez *et al*., 2010; Hoseinifar *et al*., 2011). Signor *et al*. (2010) evaluating the growth performance and metabolic response of Nile tilapia fingerlings fed diets supplemented with autolysed yeast and zinc. Dietary supplementation of partially autolyzed brewer's yeast for sub-adult

hybrid striped bass enhanced growth performance under chronic infection of mycobacteria (Li and Gatlin, 2005).

1.8.9 Significance of marine yeast SCP used as feed supplement

Marine yeast have been regarded as safe and showing a beneficial impact on biotechnological process. It provides better nutritional and dietary values indicating their potential application as feed supplements in aquaculture. Brown *et al*. (1996) evaluated all the marine yeasts characterised with high protein content, carbohydrate, good amino acid composition and high levels of saturated fats. However, there is paucity of information on marine yeasts as feed supplements and no feed formulation has been found either in literature or in market supplemented with them. This statement supported by Zhenming *et al*. (2006) reported still a lack of feed composed of single cell protein (SCP) from marine yeasts with high content of protein and other nutrients. Recent research has shown that marine yeasts also have highly potential uses in food, feed, medical and biofuel industries as well as marine biotechnology (Chi *et al*., 2009; 2010). Sajeevan *et al*. (2006; 2009a) and Sarlin and Philip (2011) demonstrates that the marine yeasts *Candida sake* served as a high quality, inexpensive nutrient source and it had proven immunostimulatory properties for cultured shrimps. This strain has been made part of the culture collection of National Centre for Aquatic Animal Health, Cochin University of Science and Technology as *Candida* MCCF 101. Over the years marine yeasts have been gaining increased attention in animal feed industry due to their nutritional value and immune boosting property.

Therefore, the present study was undertaken, and focused on the nutritional quality, optimization of large scale production and evaluation of its protective effect on Koi carp from *Aeromonas* infection.

1.9 Objectives of the proposed research were:

1) Identification of the marine yeast isolate *Candida* MCCF 101 and its proximate composition

2) Optimization of physical parameters such as pH, temperature and NaCl content in malt extract broth using Response Surface Methodology (RSM) and generation of yeast biomass in laboratory and pilot scale fermentors.

3) Optimization and scale-up of yeast biomass production in mineral based medium and comparison with that in a commercial fermentation medium.

4) The marine isolate *Candida* MCCF101 as dietary feed supplement to enhance growth, immunocompetency and survival of Koi carp (*Cyprinus carpio haematopterus*) challenged with *Aeromonas* sp. MCCB 113.

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Chapter 2

IDENTIFICATION OF THE MARINE YEAST ISOLATE *CANDIDA* MCCF 101 AND ITS PROXIMATE COMPOSITION

Contents

2.1 Introduction

Yeasts are the principal organisms that have been used for the production of single cell protein (SCP). It has a long history of human consumption and has been most commonly used in animal feed formulation and dietary supplements. Yeasts such as *Saccharomyces cerevisiae, Candida utilis, Candida tropicalis* and a few species of the genera *Hansenula, Pichia and Torulopsis* have been used for SCP production (Ravindra, 2000). Food grade yeasts have been part of fermentation industry and proved to be good sources of proteins, vitamins and growth factors. The terrestrial yeasts have been receiving great attention in scientific research and industry for over hundreds of years and their potential, well documented. They have been found economically competitive, capable of being produced in large quantities (Hardy, 2004), contain balanced amino acids and proper crude protein levels. The identification of yeast is of great practical importance in biotechnology, food technology and environmental studies.

2.1.1 Identification

Yeasts are isolated from various environments such as aquatic, marine, atmospheric and terrestrial habitats. Identification of microorganism is fundamental of biological life science. Classification systems of organisms were historically based on observable characteristics. The distinguishing morphological characteristics of a fungus are frequently too limited to allow its identification. The increased application of electron microscopy resulted in a number of significant findings. Simplified biochemical methods have also been developed based on fermentation and assimilation characteristics. These techniques contributed significantly to the advancement of yeast taxonomy, but on occasions, fluctuated with changes in environment and provided uncertain results (Yamamoto *et al*., 1991). The phenotypic approach has been largely criticized for its lack of standardized and stable terminology and for its high subjectivity. Conventional yeast identification methods based on morphological, sexual and biochemical characteristics can also be laborious and time-consuming. They can also be hard to interpret owing to the possible influence of culture medium and are therefore not suitable for precise identification. The last few years of twentieth century have seen considerable evolution in molecular biology with the development of many new techniques that can eliminate taxonomic ambiguities and simplify the identification of microorganisms (Kreger-van Rij, 1984; Barnett *et al*., 1990). Modern taxonomy is a method for recognition and registration of organism diversity. In this approach, molecular phylogenetic analysis are the basic criteria for classification of genera and species, but the cytological and morphological traits and their ecology are an integral part of taxonomic

definitions. All additional biochemical and molecular markers are also important.

2.1.1.1 Scanning Electron Microscopy (SEM)

Light microscopic observation is too limited for identification of species owing to the limited differences in morphology among yeast species. SEM can make clear identification of the surface morphological features that vary among species aiding in identification.

2.1.1.2 Molecular identification

Many methods based on molecular biology have been applied to the identification of yeasts. DNA-based methods have the advantage of being independent of gene expression. These methods include DNA–DNA hybridisation, electrophoresis of whole-cell protein, electrophoretic karyotyping, restriction fragment length polymorphism (RFLP), mtDNA-restriction analysis and DNA fingerprinting have been demonstrated to discriminate between different yeast species, but only a few species have been investigated (Lieckfeldt *et al*., 1993). Since 1990, random amplified polymorphic DNA (RAPD) has been used to identify and classify yeast species (Couto *et al*., 1994). PCR-based techniques targeting ribosomal RNA genes that can be performed with relative ease have emerged. One PCR-based technique is the study of the nucleotide divergence in the 5′-end of the large subunit (26S) ribosomal DNA gene (rDNA region D1/D2). This rDNA region is sufficiently variable to allow reliable identification of yeast species (Kurtzman and Robnett, 1997). Another technique based on the differences in the rRNA internal transcribed spacer (ITS) has been used to identify yeast species in literature. Molecular identification has several

advantages over identification using morphological and biochemical characteristics. They are independent from physiological and morphological factors like metabolism, cell composition and cellular appearance. Moreover, sequences of molecular data analysis are quite easy to assess compared to other identifications. DNA based sequence analysis is typically more stable than phenotypic differences and can vary with each isolate. Various differences in the DNA sequences of yeast between species have been exploited to effect identification (Chen *et al*., 2002). Harju *et al*. (2004) developed an easy method for DNA extraction from yeast. The basic procedure involved extraction of the DNA from the yeast culture, PCR amplification of the selected gene sequence, clean-up step of the amplified DNA, sequencing of DNA and comparison of the sequence with existing database to give the species identity.

Advances in molecular biology of yeast have led to major revisions of yeast taxonomy and classification (Kurtzman and Fell, 2006). Recent advances in phylogenomics have challenged more traditional interpretations of the evolutionary relationships existing among organisms. This study may allow further interpretation of cell biological features in an evolutionary perspective.

2.1.1.2.1 *Internal Transcribed Spacer (ITS)*

Methods based on the polymerase chain reaction (PCR) have been shown to be the most appropriate tools for rapid yeast identification. The region between the 18S rRNA and 28S rRNA genes can be amplified using specific internal transcribed spacers ITS1 and ITS4 primers (Fig.1). This region contains a highly conserved region of ribosomal 5.8S, and a variable zone which is the region of the ITSs. The ITS region, including the 5.8S

rRNA gene (coding and conserved) and two flanking variable and non-coding regions ITS1 and ITS2, show low intraspecific variability, and high interspecific polymorphism. From the conserved sequences of 18S and 28S rRNA genes at ends of the ITS region two universal primers can be obtained (Bruns *et al*., 1991). The fact that both zones are combined in the same fragment makes this a useful tool for carrying out studies at different degree of differentiation.

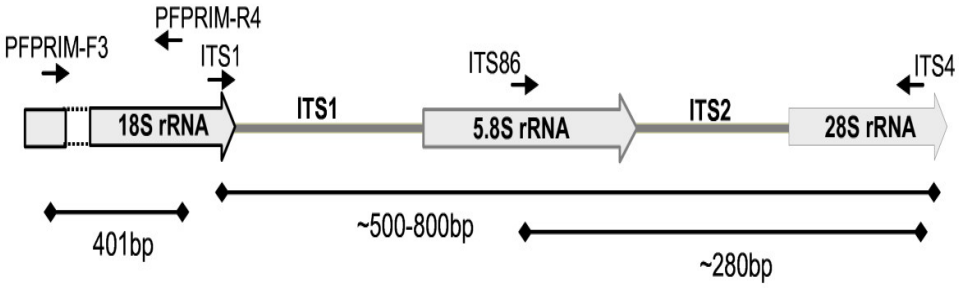

Fig.1 Schematic representation of the fungal ribosomal 18S rRNA gene and ITS regions with primer binding locations (Embong *et al*., 2008).

Amplification of the rRNA gene particularly internal transcribed spacers (ITS) of the ribosomal RNA repeats demonstrated to discriminate the different yeast species. (Turenne *et al*., 1999; Deak *et al*., 2000; Chang *et al*., 2001; Hamamoto *et al*., 2002; Li *et al*., 2003; Naumova *et al*., 2004; Hierro *et al*., 2006; Leaw *et al*., 2007).This is considered as the best tool for rapid and accurate identification of yeast isolates (Kutty and Philip, 2008). ITS primers (Forward- ITS1 and Reverse- ITS4) amplify a fragment of approximately 580 bp containing the ITS 1, 5.8s and ITS 2 regions (White *et al*., 1990). A number of sequences of rRNA and spacer have been deposited in NCBI GenBank that can be searched by basic local alignment

search tool (BLAST) (Altschul *et al*., 1990). Differentiation of closely related species requires analysis of both D1/D2 and ITS regions (Fell, 2001).

2.1.2 Nutritional Composition

The food value of microorganisms is directly related to their protein and amino acid composition, lipid, vitamin and nucleic acid content (Jay, 1996; Bekatorou *et al.*, 2006). Yeast single cell protein (SCP) has been included in animal feeds with excellent results. The search for alternative protein source of high nutritional quality has been in focus of several segments involved in aquaculture (Tacon and Barg, 1998; Anupama and Ravindra, 2000). The yeast biomass has high percentage of proteins. As an alternative protein source, yeast biomass has great potential, due to their high nitrogen content (Tacon and Jackson, 1985; Tacon, 1994; Anupama and Ravindra, 2000).They also contain about 20% non protein material which has about 8-13% purine bases, 4% pyrimidine bases, 0.5% choline, 0.5% glucosamine and other nitrogen compounds. Yeasts are rich in amino acids like lysine, threonine, and tryptophan in comparison with other protein sources of agricultural origin. Yeast proteins are easily digestible as compared to those from bacteria (Joshy and Pandey, 1999). The protein efficiency ratio and biological value of yeast protein are known to be relatively high (Munro, 1964).

The majority of the SCP are either deficient in one or more amino acids or they suffer from an amino acid imbalance (Tacon and Jackson, 1985; Kiessling and Askbrandt, 1993). The amino acid composition of yeast determines its biological value as a nitrogen source. Analysis of amino acids indicates that the yeast strains have a large amount of essential

amino acids, especially lysine and leucine which are important nutritive components for marine animals (Chi *et al.*, 2008). All the essential amino acids except methionine are present in the yeast products. The main nitrogen substrates used by yeasts during growth in food are free amino acids.

The variety of carbon sources available (CO_2, carbohydrates, hydrocarbons, lipids) makes it necessary to choose microorganisms with specific metabolic pathways. The major forms of polysaccharides in yeast cells are glycogen (storage carbohydrate), glucan and mannan (cell wall material). Glycogen and trehalose are two energy stores for the yeast cells. Trehalose plays an important protective role for the yeast cell when exposed to stresses like drying and freezing (Shima *et al.*, 1999). Several biological situations indicate that carbohydrate has an energetic function in yeast cells. Some yeast like *Candida* sp. and *Saccharomyces cerevisae* are also believed to have immunostimulatory properties by virtue of their complex carbohydrate components and nucleic acid content (Anderson *et al.*, 1995). There is increasing commercial interest in the polysaccharides of yeast cell wall i.e. their applications in food processing as thickening agents, fat substitutes and as sources of dietary fibre (Seeley, 1977; Dziezak, 1987). The ability of yeast wall glucans to stimulate the immune system (Williams *et al.*, 1992; Jamas *et al.*, 1996) and lower the serum cholesterol level (Robbins and Seeley, 1977), their antitumour activity (Bohn and Be Miller, 1995) and their potential use in cosmetics (Donzis, 1996) are noteworthy. The mannoproteins exhibit biosurfactant properties (Cameron *et al.*, 1988).

Lipids, the main constituents of cell membranes (phospholipids and sterols), function as energy storage (oils), and act as pigments (carotenoids), as well as regulator molecules in proteins (lipoproteins) and carbohydrates (Jackson, 2008). The lipid composition of microorganisms is responsive to changes in the chemical and physical properties of the environment. Majority of yeast accumulate lipids usually as triglycerides containing a high percentage of palmitic acid, particularly near the end of the growth cycle. Among the environmental factors that have been reported to affect the lipid composition of microorganisms are growth rates, composition of the medium, growth temperature, and dissolved O_2 tension in the culture. Since, with most organisms, the bulk of the cell lipids are in membranes, it is likely that these environmentally induced changes in lipid composition are of major physiological significance (Hunter and Rose, 1972). Dietary lipids are important sources of energy and fatty acids that are essential for normal growth and survival of fish. Sanderson and Jolly (1994) observed that yeast is a rich carotenoid source for salmonid fish. Yeast and moulds have a fatty acid composition more closely related to that of plants. Yeast cells can consume metabolites other than glucose to generate energy, including fatty acids.

The microbial identification method based on Fatty Acid Methyl Esters (FAME) analysis can be used to characterize microbial communities (Zelles, 1999; Peltroche-Liacsahuanga *et al.*, 2000). The system analyses long-chain fatty acids containing 9–20 C atoms, identifying and quantifying the FAMEs of microorganisms. In yeast, different classes of fatty acids are involved in stabilizing or resolving highly curved membrane structures, such as those formed as intermediates during membrane budding and

fusion (Schneiter and Kohlwein, 1997). Straight chain fatty acids are the major constituents of phospholipid membranes. Composition of the growth medium, availability of oxygen, temperature, pH and the age of the culture can each affect the distribution of the fatty acids. Fatty acids with C_{16} and C_{18} atoms are generally predominant in yeast. Bell *et al.* (2003) reported that the lipid component of the diet must provide an adequate amount of essential fatty acids for growth as well as energy. The polyunsaturated fatty acids (PUFA), major components of cell membranes, are responsible for the eicosanoid production and release and provide structural integrity to the membrane as well. Poly unsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA) are essential for normal growth, moulting and maturation of shrimp and various crustaceans (Xu et al., 1994; Sargent et al., 1999; Wouters et al., 2001a, b).

Nucleic acids are a necessary component of all cells but present in relatively high levels in rapidly dividing cells. In brewer's yeast, nucleic acid nitrogen is present mostly in the form of RNA and represents about 20 % to 25 % of the nitrogen (Rumsey *et al.*, 1991b). Dietary nucleotides have beneficial effects, as they positively affect the immune system, hepatic function, lipid metabolism, disease resistance, development of small intestine and growth (Carver and Walker, 1995; Cosgrove, 1998; Carver, 1999; Burrells *et al.*, 2001; Low *et al.*, 2003; Li *et al.*, 2004). The nucleic acid concentration in single cell protein is the only reason of concern about its intake as dietary food for humans. Mammals in general excrete allantoin, a non-toxic material which is formed from uric acid, the toxic intermediate of nucleic acid metabolism, in single step in the presence of the enzyme uricase. Enzyme uricase is absent in humans and high quantities of nucleic acids in

diet may result in liver damage. Uricase is present in fish (Joshy and Pandey, 1999) and crustaceans. The marine yeasts with high levels of nucleic acids could be used as a feed to aquaculture animals that produce uricase. Sakai *et al.* (2001) reported that the nucleotides from brewer's yeast RNA were capable of enhancing the phagocytic and oxidative activities of kidney phagocytic cells, serum lysozyme in common carp as well as resistance to *Aeromonas hydrophila*.

Yeast is also a good source of vitamins, especially vitamin B. Among the vitamins and other cofactors accumulated or synthesized by yeast are thiamine (vitamin B_1), nicotinic acid containing co-enzymes NAD and NADP, pyridoxine (vitamin B_6), pantothenic acid, cyanocobalamin (vitamin B_{12}), biotin (also known as vitamin B_7, Vitamin H or cofactor R), folic acid (also known as vitamin B_9, vitamin BC or folate) and riboflavin (vitamin B_2) (Halasz and Lasztity, 1991). Yeasts are also important source of flavins derived from riboflavin (Vitamin B_2), the water soluble ascorbic acid (vitamin C) and multiple forms of co-enzyme Q (Hancock and Viola, 2001). B group vitamin riboflavin can improve growth, feed utilization, body composition and intestinal enzyme activities of juvenile Jian carp (Li *et al.*, 2010b). Appropriate levels of minerals in diets not only improve animal welfare but they also prevent diseases (Breck *et al.*, 2003). The nutritive value of yeast products differs according to its type.

2.1.2.1 Nutritional value of yeasts biomass in aquaculture

Among the alternative protein sources or supplements that hold particular promise in aquaculture are the single-cell protein (SCP) from microorganisms (Bhattacharjee, 1970). Yeast SCPs are playing a greater role in the evolution of aquaculture diets. With excellent nutrient profiles

and capacity to be mass produced economically, SCPs have been added to aquaculture diets as partial replacement of fishmeal (Coutteau and Lavens, 1989; Olvera-Novoa *et al.*, 2002; Li and Gatlin III, 2003). Most feasible among them are the yeast species recently being exploited as aquafeeds (Tacon, 1994; Padua *et al.*, 2000; Olvera-Novoa *et al.*, 2002; Lara-Flores *et al.*, 2003; Lim *et al.*, 2005; Hisano *et al.*, 2007). Yeast has been evaluated as a growth promoter and disease preventer in fish diets. Yeast and yeast by-products from brewing industry are natural diet additives that positively influence non-specific immune responses (Siwicki *et al.*, 1994; Anderson *et al.*, 1995) and growth (Rumsey *et al.*, 1991b; Oliva-Teles and Goncalves, 2001) of some fish species. Industrial yeast is commonly used in aquaculture, either alive as feed, or after processing, as a feed ingredient (Stones and Mills, 2004). Yeast showed promising results in growth improvement (Li and Gatlin III, 2003) health and immune response in fish (Li and Gatlin III, 2004) as a functional food. These additional properties enabled yeast to be included in animal diets at lower concentrations (Signor *et al.*, 2010).

Candida sp., *Hansenula* sp., *Pichia* sp., and *Saccharomyces* sp. have special importance as component in fish feeds (Ebrahim and Abou-Seif, 2008). Sajeevan *et al.* (2009a) reported that the use of biomass of *Candida* in diets would confer better protection against microbial infection, rather than the yeast cell wall extract, glucan. The use of *Saccharomyces cerevisiae* has improved the growth performance and feed efficiency in tilapia cultivation (Lara-Flores *et al.*, 2003). Ozorio *et al.* (2010) reported replacing 50% fishmeal by yeast in Pacu (*Piaractus mesopotamicus*) diets successfully improved feed efficiency and growth performance, and

reduced nitrogen losses. Marine yeasts have potential uses in food, feed and medical industries (Chi *et al*., 2009) as well as in marine biotechnology. In bivalve aquaculture Brown *et al.* (1996) evaluated the possibility of using marine yeasts *Debaromyces hansenii* ACM 4784, *Dipodascus capitatus* ACM 4779, *Dipodascus* sp. ACM 4780 as feed supplements.

2.2 Materials and methods

2.2.1 Yeast Strain

Candida sake S165 was isolated from coastal waters off Cochin, and morphological and biochemical characteristics analysed (Sarlin, 2005; Sajeevan, 2006). This culture was received from the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Kerala, India and subsequently deposited in the Microbial Culture Collection of National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology, Kerala, India as *Candida* MCCF 101.

2.2.1.1 Culture Medium

The solid medium used for culturing *Candida* MCCF 101 was malt extract agar with the following composition:

Malt extract agar

Malt extract	:	17 g
Peptone	:	3 g
Agar	:	20 g
Sea water	:	1000 ml
pH	:	5.5

2.2.2 Inoculum Preparation

Malt extract agar slants were prepared and sterilized by autoclaving. The selected yeast strains were streaked on to malt extract agar slants and incubated at room temperature (28± 2^{o}C) for 24 hrs. Twenty four hour old lawn culture of the yeast was harvested into sterile seawater of salinity 20gL^{-1} Its absorbance was adjusted to 0.1 at 540 nm in a UV-VIS spectrophotometer (UV-1601, Shimadzu Corporation, Tokyo, Japan) using sterile sea water which provided a cell count of 5.57×10^{5}cfu mL^{-1} to be used as the inoculum (0.1mL per 100mL medium) for further experiments.

2.2.3 Morphological characterization using scanning electron microscopy

An aliquot of 1.5 ml of yeast culture was centrifuged at 8000 RPM in a refrigerated centrifuge for 15 min. The pellet was washed with sterile saline of 5 $^{0}/_{00}$ and fixed in 0.5 ml of 2.5 % glutaraldehyde prepared in sterile saline at 4^{o}C overnight. The pellet was washed repeatedly with saline, dehydrated through an acetone series of 70-100% and kept overnight in a dessicator. The particle was spread on SEM stubs, dried in critical point drying apparatus, platinum coated and observed under JEOL Analytical Scanning Electron Microscope, JSM 6390 LV, Tokyo, Japan (Sophisticated Test and Instrumentation Centre, CUSAT, Cochin-22).

2.2.4 Molecular identification

2.2.4.1 DNA based identification

2.2.4.1.1 *DNA isolation*

Yeast genomic DNA was isolated according Harju *et al*., 2004. An aliquot of 1.5 ml of the overnight culture of yeast grown at 30^{o}C in YPD (1% yeast

extract, 2% peptone and 2% dextrose) was centrifuged at 2000 x g for 5 min and the cell pellets were resuspended in 200µl of lysis buffer (2% Triton X- 100, 1% SDS, 100mM NaCl, 10mM Tris-HCl, 1mM EDTA of pH 8). The tubes were placed in -80^{o}C freezer for 5 min, taken out and then immersed in a 95^{o}C water bath for 1 min to thaw quickly. The process was repeated once and the tubes were vortexed for 30 sec. An aliquot of 200 µl of chloroform was added and vortexed for 2 min to precipitate proteins and then centrifuged for 3 min at room temperature at 20000 x g. The aqueous layer obtained was transferred to a tube containing 400 µl of ice-cold 100 % ethanol. The tubes were incubated overnight at -20^{o}C to precipitate DNA and centrifuged at 20000g for 20 min at 4^{o}C. Supernatant was removed and DNA pellets were washed twice with 0.5 ml of 70 % ethanol followed by air-drying at room temperature. DNA was resuspended in 20 µl of TE buffer (10 mM Tris, 1mM EDTA of pH 8) and stored at 4^{o}C for future use. The isolated DNA was quantified spectrophotometrically (Abs_{260}) and the purity of DNA was assessed by calculating the ratio of absorbance at 260 nm and 280 nm (Abs_{260}/Abs_{280}), the value of which determined the amount of protein impurities in the sample. Electrophoresis was done using 0.8 % agarose gel and the DNA marker used was that of 1 kb (New England Biolabs, USA).

Concentration of DNA was calculated using the following formula:

Conc. of DNA (µg/ml) = Abs. at 260 nm x 50 x dilution factor.

2.2.4.1.2 *rRNA gene sequence analysis*

The ITS (Internal Transcribed Spacer) region was amplified by PCR using the forward primer ITS 1-5' TCC GTA GGT GAA CCT GCG G 3' and reverse primer ITS 4- 5' TCC TCC GCT TAT TGA TAT GC 3' described by White *et al*. (1990). A fragment of approximately 580 bp containing ITS 1, 5.8 S and ITS 2 regions was amplified as described below. An initial denaturation step was performed at 95^{o}C for 5 min. Amplification was made through 30 cycles, each consisting of a denaturation at 94^{o}C for 1 min, annealing at 56^{o}C for 45 sec, extension at 72^{o}C for 1 min followed by a final extension at 72^{o}C for 10 min. The PCR product was analyzed together with a molecular weight ladder by electrophoresis on a 1% agarose gel.

a) Cloning of pGEM-T easy vector system

The PCR product was cloned into pGEM-T easy vector system (Promega, USA) and sequenced using the primer walking service of Microsynth AG Switzerland. All sequence obtained were matched with the database in GenBank using the BLAST algorithm and the ribosomal data base project (RDP) release 9 (Altschul *et al*., 1990; Lee *et al*., 2003b). Fresh PCR product of ITS region was used for cloning into the pGEM-T Easy vector (Promega, USA). The ligation mix (10 µl) consisted of 5 µl ligation buffer (2X), 0.5 µl of the vector (50 ng/ µl), 3.5 µl of PCR product (600 ng/ µl) and 1 µl of T4 DNA ligase (3U/ µl). It was incubated at 4^{o}C overnight.

The entire ligation mix was used for transformation into JM 109 high efficiency competent cells of *Escherichia coli* (transformation efficiency

≥ x10^8 cells/ µg DNA). The ligation mix was added to 10 mL glass tube previously placed in ice to which 50 µl of competent cells were added and incubated on ice for 20 min, a heat shock at 42^{o}C was given for 90 seconds to facilitate the entry of recombinant DNA to the host cells and the tubes were immediately placed on ice for 2 min. An aliqot of 600 µL of SOC medium (2% tryptone, 0.5% yeast extract,10 mM sodiumchloride, 2.5mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose per litre) was added and incubated for 2 hrs at 37^{o}C in an incubator shaker at 250 rpm. The transformation mixture (200 µl) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), IPTG (100 mM), and X-gal (80 µg/ml). The plates were incubated at 37^{o}C overnight. The clones/transformants were selected using the blue/white screening. Successful cloning of an insert into the pGEM-T easy vector interrupts the coding sequence of β-galactosidase; recombinant clones can be identified by color screening on indicator plates. The white colonies were selected and streaked on LB-Amp + X-gal + IPTG plates and incubated overnight at 37^{o}C. Colony PCR was done to confirm the presence of the insert DNA (DNA fragment to be cloned). All the individually streaked colonies were tested with colony PCR using universal vector primers T7 (5'-TAATACGACTCACTATAGGG- 3') and SP6 (5'GATTTAGGT GA CACTATAG-3') to confirm the presence of the gene of interest and electrophoresis was done on 1 % agarose gel prepared in 1X TBE buffer and stained with ethidium bromide.

White colonies (template) picked from the transformed plate were dispensed into the PCR reaction mix (25 µl) containing 2.5 µl 10X PCR buffer, 2.0 µl of 2.5 mM dNTPs, 1 µl of 10 pmol/ µl of T7 and SP6 primers,

0.5 U of taq polymerase and the remaining volume was made up with Milli Q. The thermal cycling conditions were as follows: After an initial denaturation at 95^{o}C for 5 min, amplification was made through 35 cycles, each consisting of a denaturation at 94^{o}C for 15 sec, annealing at 57^{o}C for 20 sec, extension step at 72^{o}C for 1 min and a final extension at 72^{o}C for 10 min following which the temperature was brought down to 4^{o}C.

b) Plasmid extraction and purification

Plasmid extraction and purification was done using 'GenElute HP' plasmid miniprep kit (Sigma). Cells were harvested by centrifuging 2 ml of overnight grown recombinant *E.coli* culture at 16000 x g for 20 min. The pellet was resuspended in 200 µl resuspension solution with RNase. Lysed the resuspended cells by adding 200 µl of the lysis buffer. This suspension was immediately mixed using gentle inversion until the mixture became clear and viscous. The cell debris was precipitated by adding 350 µl of the neutralization buffer. The tube was gently inverted and the cell debris was pelleted by centrifuging at 16000 x g for 10 minutes. Column was prepared by inserting a Gen Elute HP Miniprep Binding column into a provided microcentrifuge tube. Contaminants were removed by spin wash step. Transferred the column to a fresh collection tube and added 100 µl of elution solution (10 mM Tris-HCl) to the column and centrifuged at 16000 g for 1 min. The DNA present in the eluate (plasmid DNA) was stored at -20^{o}C.

Nucleotide sequencing was performed using ABI PRISM 3700 Big Dye Sequencer at Microsynth AG, Switzerland. The primers used were T7 and SP6. Sequenced DNA data was complied and analyzed. The sequence obtained was first screened for vector regions using 'VecScreen' system accessible from the National Centre for Biotechnology Information (NCBI).

The Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al*., 1990) was used to search the GenBank database for homologous sequences (http:// www.ncbi.nlm.nih.gov/). The sequences were multiple aligned using the programme Clustal W (Thompson *et al*., 1994). The aligned ITS-rDNA gene sequences were used to construct a phylogenetic tree using the neighbour-joining (NJ) method (Saitou and Nei, 1987) using the MEGA4 package (Tamura *et al*., 2007). Bootstrap analysis was based on 1000 replicates.

2.2.5 Proximate Composition of the yeast biomass

2.2.5.1 Biochemical Analysis

Biochemical composition of the yeast biomass was analysed to assess their nutritional quality. Cells were harvested from the fermentor by centrifugation at 3500 rpm for 30 min at 4ºC. The pellet was collected and washed repeatedly with saline (0.05%NaCl), and dried in an incubator at 60ºC until the constant weight was reached. The dried cells were powdered using grinder and used for biochemical analysis.

Protein content was calculated from the nitrogen content (%N × 6.25) analysed by micro kjeldahl method (Lang, 1958). Carbohydrate content was estimated using Anthrone method (Hedge and Hofreiter, 1962) and lipid by Sulpho - phosphovanillin method (Barnes and Blackstock, 1973). Nucleic acid analysis was done by colorimetric reaction with orcinol described by Endo (1970) for RNA, and Diphenylamine method by Burton (1977) for DNA. Determination of total dietary fiber was by detergent method (Goering and Van Soest, 1970). Ash content was determined by incineration at 550^{o}C in a muffle furnace for 5hrs and moisture content by drying in an oven at 80^{o}C to constant weight.

2.2.5.1.1 *Protein determination*

The protein content of the biomass of *Candida* MCCF 101 was estimated as total nitrogen by micro kjeldahl method. The crude protein content calculated from the equation total nitrogen x 6.25 (Lang, 1958).

2.2.5.1.2 *Total Carbohydrates*

Total carbohydrate in the yeast biomass was determined spectrophotometrically by Anthrone method (Hedge and Hofreiter, 1962). Sample (100 mg) was hydrolysed in a boiling water bath for 3 hrs with 2.5 N HCl and cooled and neutralised with solid Na_2CO_3 until the effervescence ceased. The volume was made up to 100 ml by adding distilled water and centrifuge. To the supernatant added 4 ml of anthrone reagent (200 mg anthrone in 100 ml ice-cold Conc. H_2SO_4) and heated for 8 minutes in a boiling water bath, cooled rapidly and read the green colour at 630 nm. Plot glucose concentrations 0 to 100µg/mL were used for the standard curve and from this curve, concentration of carbohydrate was calculated. Amount of carbohydrate was expressed as g/100gm dry weight.

2.2.5.1.3 *Estimation of Lipids*

The lipid content of the yeast biomass was analysed by Phosphovanillin method following chloroform-methanol extraction of the sample (Barnes and Blackstock, 1973). Sample 500mg was mixed well with 10ml of chloroform:methanol solution (2:1) in a homogeniser and filtered the homogenate through Whatman No. 1 filter paper, added 2 ml of 0.9 % NaCl and shaken well. The mixture transferred to a separating funnel, allowed to stand overnight at 4^oC. The lower phase removed (contained all the lipids) and adjusted the volume to 10ml by the addition of chloroform. An aliquot of

0.5 ml of the extract was taken in a clean tube and allowed to dry in vacuum dessicator over silica gel. After drying, 0.5 ml of Conc. H_2SO_4 was added to the sample, mixed well to dissolve and placed in a boiling water bath for 10 min and cooled to room temperature. To 0.2 ml of the acid digest 5 ml of vanillin reagent was added and incubated for 30 min and measured the absorbance at 520nm. Cholesterol (0.0 to 1.0mg/mL) prepared in (2:1) chloroform: methanol served as standard.

2.2.5.1.4 *Estimation of Nucleic acids*

DNA estimation was done by diphenylamine method and RNA by Orcinol method (Kochert, 1978). Ribose of RNA or deoxyribose of DNA reacts with orcinol reagent forming a bluish green colour at 660 nm. But only DNA gives colour with diphenylamine reagent at 600 nm. Diphenylamine produces a blue colour by reaction with deoxyribose moiety in DNA. From the difference in colour intensity by two methods, composition of the mixture can be calculated.

2.2.5.1.5 *Determination of fibre content*

The detergent method of Goering and Van Soest (1970) with a modification by Van Soest and Mc Queen (1973) was used to estimate the fibre content. A known quantity of yeast biomass was dried at 60^{o}C for 8 hrs and weighed again. It was ground to pass through a 1mm sieve. A detergent solution was prepared by mixing sodium lauryl sulphate (3% at final concentration), disodium ethylene-diamine tetraacetate dehydrate (1.86% at final concentration), anhydrous disodium hydrogen phosphate (0.456% to final volume), sodium borate decahydrate (0.681% to final volume) and 2-ethoxy ethanol (1ml per 100 ml solution). An aliquot of 100mL of this detergent solution was added to 1g of sample. Decalin (2ml

per 100 ml of detergent solution) and sodium sulphite (0.5% to final concentration) was adds to the mixture and placed in a Berzelius beaker for refluxing. The mixture was heated to boiling and refluxed for one hour. Cooled and washed with hot water (80^{o}C) and centrifuged. The mixture was washed with water and acetone. The residue was collected, dried at 105°C for 8 hours and weighed. The content of total dietary fibre was expressed as g/100g of the dry weight of the sample.

2.2.5.1.6 *Determination of ash content*

Ash content was determined by incineration of yeast biomass in a silica crucible at 550^{o}C in a muffle furnace for 5 hrs. Weight of the residue in the crucible was the ash content.

2.2.5.1.7 *Determination of moisture*

The moisture content of wet yeast biomass was measured by drying 10g of freshly harvested yeast biomass at 105^{o}C for 18 hrs or till constant weight was attained and calculating the difference in weight.

2.2.5.1.8 *Amino acid analysis*

Separation of amino acids was accomplished using HPLC-LC (Shimadzu 10 AS automatic amino acid analyzer) as per the procedure of Ishida *et al.* (1981). Qualitative and quantitative estimation of each amino acid was accomplished by comparing with standard chromatograms constructed using known concentrations of amino acids and presented as percentage of total protein. This procedure was applied for the estimation of all amino acids except tryptophan. The amino acid tryptophan is not stable to acid digestion in the presence of oxygen and was estimated

separately following digestion under alkaline conditions with thioglycolic acid reagent (Sastry and Tummuru, 1985).

a) Sample preparation

Yeast biomass (100 mg dry weight) was taken in a heat stable test tube; added 10 ml of 6N HCl and heat sealed the tube after filling with pure nitrogen gas. The hydrolysis was carried out at 110^{o}C for 24 hrs. After hydrolysis, the test tube was opened and the contents were removed quantitatively and filtered into a round bottom flask through Whatman filter paper No.42. The filter paper was washed 2-3 times with distilled water. Flash evaporated the contents of the flask to remove all traces of HCl. The process was repeated 2-3 times with distilled water. Dissolved the residue and made the volume to 10 ml with 0.05 M HCl.

b) HPLC Analysis

The sample obtained from the above step was filtered through a membrane filter of 0.45 µm and injected 20 µl to an aminoacid analyzer equipped with sulphonated polyvinyl styrene cation exchange column and fluorescence detector. The mobile phase of the system consisted of two buffers, A and B. A gradient system was followed for the effective separation of amino acids. The oven temperature was maintained at 60^{o}C. The amino acid analysis was done with non-switching flow method and fluorescence detection after post-column derivatization with o-phthalaldehyde. Amino acid standards were run to calculate the concentration of amino acids in the sample.

c) Estimation of tryptophan (Sastry and Tummuru, 1985)

Since tryptophan content could not be obtained through HPLC, estimation was done separately. About 200-250 mg of sample (dry weight)

was hydrolyzed with 10 ml of 5 % NaOH at 110^{o}C for 24 hours in a sealed tube filled with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6 N HCl using phenolphthalein indicator. The volume was made up to 100 ml with distilled water. The solution was then filtered through Whatman No.1 and the filtrate was used for estimation. To a test tube containing 4ml of 50 % H_2SO_4, 0.1 ml of 2.5 % sucrose and 0.1 ml of 0.6 % thioglycolic acid were added. These tubes were kept for 5 min in water bath at 45-50^{o}C and cooled. The sample was then added to these test tubes. A set of (0.1 to 0.8 µg) standard was treated similarly. The volume was made up to 5ml with 0.1 N HCl and allowed to stand for 5 minutes. The absorbance was measured using UV-Vis spectrophotometer at 500 nm.

The amount of Tryptophan in the sample was calculated from the equation

$$\text{Tryptophan} = \frac{\mu\text{g of Tryptophan} \times \text{volume made up} \times 100 \times 16}{\text{Sample taken for colour development} \times \text{Weight of sample} \times 1000 \times \%\text{ of } N_2}$$

d) Estimation of total free amino acid

Total free amino acid was estimated by Ninhydrin method (Yemm and Cocking, 1955). Ninhydrin decarboxylases the α-amino acids resulting in a bluish purple product which can be measured spectrophotometrically. Mixture of glycine and glutamic acids were used as standard in this experiment. Added 0.5 ml of citrate buffer and 1.2 ml of ninhydrin reagent to 1 ml of sample. The sample was prepared by macerating 10mg yeast (dry weight) in 80% ethanol and transferred in to a centrifuge tube, centrifuged at 4500 x g for five minutes and collected the supernatant. Covered the test tube with the supernatant with a piece of paraffin film to

avoid the loss of solvent due to evaporation. With gentle stirring, heated the solution at 100^{o}C for 15 min. After cooling to room temperature in a cold water bath added 2.3ml of 70% ethanol to each tube and recorded the absorbance at 570nm in a spectrophotometer.

2.2.5.1.9 *Fatty acids analysis*

For fatty acid analysis, gas chromatograph with flame ionization detector (GC-FID) was employed (Agilent Technologies, model 6890).

a) Sample preparation

Candida MCCF101 was streaked on to Sabouraud dextrose agar plate employing the quadrant streak method. It was incubated overnight at 28^{o}C. Quadrant streak method was designed to dilute the inoculum so that quadrant 4 will contain well-isolated colonies to serve as a check for purity. Colonies were harvested from the most diluted quadrant (Quadrant 3) exhibiting late log phase along the streaking axis. This area of harvesting typically yields the most stable fatty acid compositions since the inoculum has been diluted enough to result in abundant growth of colonies without limiting nutrient supply. Yeast colonies weighing up to 100 mg were carefully harvested using a small diameter wire loop.

The yeast cells were harvested from the culture plates and the whole cell fatty acid methyl esters (FAMEs) were obtained by saponification, methylation and extraction into hexane:methyl *tert*-butyl ether. A 25m (length) x 0.2 mm ID x 0.33µm film thickness, cross linked by 5% phenylmethyl silicone fused silica capillary column was used to separate the fatty acids.

b) Saponification

A strong methanolic base combined with heating, killed and lysed the cells. Fatty acids were cleaved from cell lipids and were converted to their sodium salts. A saponification reagent was prepared by dissolving 15g NaOH in 100mL of methanol:distilled water mixture (1:1). Methanolic base (1ml) pipetted out into the culture tube. It was tightly sealed with a clean Teflon-lined screw-cap. The tube was vortexed for 5-10 sec, placed in a circulating water bath at 95-100^{o}C and after 5 minutes removed from the water bath and cooled slightly without loosening the cap. The tube was vortexed again for 5-10 sec and returned to the water bath and heated in the water bath for an additional 25 min. After a total of 30 min of saponification, the tube was removed and placed in a pan of cold tap water and cooled.

c) Methylation

Methylation converts the fatty acids (as sodium salts) to fatty acid methyl esters, which accelerates the volatility of the fatty acids for GC analysis. A methylation reagent was prepared by mixing 325mL 6 N HCl and 275 ml HPLC grade Methanol. The tube was uncapped, and 2mL methylation reagent was added. It was tightly capped and vortexed the solution for 5-10 seconds heated in an 80^{o}C water bath for 10 minutes. It was removed and quickly cooled to room temperature by placing in a tray of cold tap water. The tube was shaken to speed up the cooling process.

d) Extraction

Fatty acid methyl esters were removed from the acidic aqueous phase and transferred to an organic phase with a liquid-liquid extraction procedure. An extraction solvent was prepared by mixing HPLC grade hexane and methyl

tertiary butyl ether (1:1). The tube was uncapped, and 1.25 ml of the extraction solvent was added to the tube. It was sealed tightly and placed in a laboratory rotator and gently mixed end-over-end for 10 minutes. The tube was uncapped, and using a Pasteur pipette, removed and discarded the lower aqueous phase.

e) Base Wash

A dilute base solution was added to the sample preparation tubes to remove free fatty acids and residual reagents from the organic extract. Residual reagents will damage the chromatographic system, resulting in tailing and loss of the hydroxyl fatty acid methyl esters. This base wash was prepared by dissolving 10.8g NaOH and 40g NaCl in 1L deionized distilled water. Added 3 ml of the base wash to the tube. It was tightly capped and gently rotated end-over-end for 5 minutes.

The upper solvent phase was removed and placed in a sample vial suitable for automatic sampler mounted on the gas chromatograph. Uncapped the tube and using a clean Pasteur pipette, transferred about 2/3 of the upper organic phase to a clean GC sample vial. A cap was crimped onto the sampler vial, loaded the automatic sampler and it was run. An aliquot of 2µl sample was injected. The run lasted for 20.7 minutes. The initial temperature 170ºC was increased to 31ºC at the rate of $40^{o}C\ min^{-1}$ and held for 1.5 min. Hydrogen was used as the carrier gas at a constant flow rate of 1.3 L min^{-1}. The peaks were analyzed by using the software Sherlock (MIDI, Inc., USA) to identify the relative amounts of fatty acids in the sample and were expressed as percentage of the total fatty acids. Identification of the peaks was accomplished by comparison of retention times to those of authentic standards.

2.3 Results

2.3.1 Morphology

Most of the cells were elliptical in shape. The scars from where buds were detached were permanently seen on cells and that gave some of the cells a club shape. The cells exhibited irregular surface features probably due to their mannoprotein, fibrous β-1, 3 glucan and chitin components. The average size of the cell is 5µm (Fig. 2).

2.3.2 Identification of the isolate by ITS region sequence analysis

DNA was isolated (Fig.3) and amplified using universal primers of ITS region yielding a PCR product of 500bp (Fig.4).Which cloned in to pGEMT easy vector and transformed *Escherichia coli* (JM109) and confirmed of the insert by colony PCR using T7 and SP6 vector primers which produced a product of 650bp (Fig.5). Plasmid from the transformed organisms was extracted and partially sequenced using T7 and SP6 vector primers. The nucleotide sequences (Fig.6) determined in this study was deposited in GenBank data base of NCBI (www.ncbi.nlm.nih.gov) and assigned the accession no.FJ652052 (Appendix1). The sequence from the clones containing ITS region when matched with the GenBank data base showed 98% similarity to *Candida* sp. S27, and 97.8 % similarity to *Candida aquae-textoris* strain ATCC 20145. Phylogenetic tree constructed based on sequence of ITS region showed that *Candida* MCCF 101 (Fig.7) was positioning itself between *Candida* sp. S27and *Candida aquae-textoris* strain ATCC 20145.

2.3.3 Proximate composition

Results of the analysis revealed the protein content of the yeast biomass as 30 ± 1.63%, carbohydrate 36.25 ± 1.25%, lipid 1.52 ± 0.04%,

nucleic acid 12.05±0.05%, dietary fiber 10.05 ± 0.50%, ash 6.67 ± 0.94% and moisture 77 ±0.50%.

2.3.3.1 Amino acid analysis

Candida MCCF 101 contained essential and non-essential amino acids, such as, aspartic acid (8.67%), threonine (4.53%), serine (4.87%), glutamic acid (14.84%), proline (3.61%), glycine (4.51%), alanine (7.79%), valine (7.81%) methionine (1.66%), isoleucine (6.27%), leucine (14.31%), tyrosine (3.99%), phenylalanine (4.88%), histidine (1.99%), lysine (4.31%), arginine (2.56%) when estimated by HPLC. Tryptophan which was estimated separately accounted for 6.5% of total protein of the cell biomass (Table1). Among the amino acids glutamic acid and leucine were of highest concentration (Fig. 8).

2.3.3.2 Total free amino acid

Total free amino acid (FAA) content estimated was 16.16% of total amino acids of the dry mass of the yeast.

2.3.3.3 Fatty acid composition

Fatty acid profile exhibited long chain fatty acids (up to 18-carbon atoms) along with polyunsaturated fatty acids (PUFA). Fatty acids recorded were lauric acid (0.54%), myristic acid (2.17%), pentadecanoic acid (0.68%), palmitoleic acid (6.37%), palmitic acid (15.66%), margaroic acid (4.25%), margaric acid (0.47%), linoleic acid (22.45%) and stearic acid (18.0%) of the total lipids of the dry yeast biomass (Table 2). Among them linoleic acid, stearic acid and palmitic acid were dominant (Fig. 9).

2.4 Discussion

Guarro *et al*. (1999) reported that the identification of yeasts has been traditionally based on morphological characteristics, but due to their poor differentiation, biochemical and electron microscopic studies were largely used. Modern taxonomy is a method for recognition and registration of organism diversity. In this approach, molecular phylogenetic analysis are the basic criteria for classification of genera and species, but the cytological and morphological traits and their ecology are an integral part of taxonomic definitions. Therefore, systematics has turned increasingly to molecular approaches to distinguish and identify yeast species and to develop a system of classification based on phylogeny. A molecular biology technique, especially the analysis of rRNA sequences, is currently used for reliable phylogenetic studies, which enable a more natural classification system to be established.

Candida MCCF 101 produced elliptical cells or club shaped cells of 5µm diameter which made it distinct from other *Candida* species *C. albicans* and *C.utilis.* Its budding was similar to what was observed in *Saccharomyces cerevisiae* and it has a scar where budding occurred. *Candida* MCCF101 produced 2.15×10^{8} cfu mL^{-1} when grown in liquid broth. It has smell of chocolate or fruit, when biomass was dried. Due to the larger size (5µm) of the marine yeast (*Candida* MCCF 101) the cells settled when kept without agitation.

The nucleotide sequences determined in this study was deposited in GenBank and assigned the Accession No.FJ652052. The phylogenetic tree generated was based on 20 aligned sequences of yeast ITS region from

GenBank data base where the genus *Candida* could be identified as the common ancestor. Accordingly *Candida* MCCF 101 showed 98% similarity to *Candida* sp.S27, and 97.8 % similarity to *Candida aquae-textoris* ATCC 20145. Hence, it is suggested that *Candida* MCCF 101 is closely related to *Candida* sp.S27.

Proper nutrition has long been recognized as a critical factor in promoting normal growth and sustaining health of fish and shell fish. In recent years, marine yeast has been used as a diet additive for various animals as they have higher concentration of essential amino acids such as lysine, methionine and leucine and served as immunostimulants. Various studies conducted with fishes and shrimps have shown that yeast and yeast derivatives are effective growth enhancers (Scholz *et al*., 1999; Li and Gatlin III, 2003; 2004).

The usefulness of yeast biomass as single cell protein is principally judged based on its proximate composition which includes protein, carbohydrates, fat, nucleic acid and amino acids composition. The protein content (30.1%) is found to be comparable with that of *Candida utilis* (31.3%) (Joshi and Pandey, 1999) and *Candida tropicalis* (28.1%) (Pessoa *et al*., 1996) which are traditionally used as fish feed. Fish and shell fish require dietary protein which contains a balanced source of essential and non essential amino acids for their growth, maintenance, and reproduction (Srivastava *et al*., 2006; Guillaume, 1997). Shiau *et al*. (1991) found evidence that the dietary protein levels potentially influence the culture of *Penaeus monodon* when reared in low salinity environments.

Guillaume (1997) suggested that carbohydrates are very efficient energy sources in standard metabolism, muscular energy expenditure and other processes requiring ATP. *Candida* MCCF 101 contains 36.25% carbohydrate which may satisfy the energy needs of cultured crustaceans. Dietary lipids are a source of essential fatty acids, phospholipids, sterols and carotenoids required for growth, survival and the normal metabolic functions of penaeid shrimp (Blazer, 1992; Gonzalez and Velazquez, 2002). Phospholipids are also essential for crustaceans which serve as second messengers in cell signalling and also involved in lipid metabolism (Teshima, 1997). Even though shrimps are able to synthesize their own phospholipids, the levels are well below the dietary requirements. *Candida* MCCF 101 contains 1.52% lipids which may satisfy this lipid requirement.

Candida MCCF 101 was found to have 12.05% nucleic acid content, experimentally proven to be immunostimulatory. Chuo *et al*. (2005) showed that signal transduction in the prophenoloxidase activating system of *Macrobrachium rosenbergii* and intracellular phenoloxidase activity in haemocyte lysate supernatant (HLS) could be increased after treatment with CpG oligonucleotides which demonstrated the role of nucleotide content of yeast in immunostimulation. Though high nucleic acid content is undesirable for human consumption (Maul *et al*., 1970) it is harmless to most animals including crustaceans (Ohta *et al*., 1971; Zee and Simrad, 1975). A growing number of reports have indicated that nucleotides are capable of enhancing immune responses and/or diseases resistance in several fish species as well as in shrimps (Li and Gatlin III, 2004; Chuo *et al*., 2005). Ash content of *Candida* MCCF 101(6.67%) was closer to the range (7-8%) described in the literature (Paul *et al*., 2002; Rajoka *et al*., 2004).

The essential amino acids such as valine, isoleucine, phenylalanine, histidine, methionine, arginine, threonine, lysine, leucine and tryptophan are not synthesized in fish and shrimps which are the absolute dietary requirements (Cowey and Forster, 1971; Nose, 1974). All these amino acids are present in *Candida* MCCF101 making it a good feed supplement.

Candida MCCF 101 has satisfactory level of FAAs which have been found to be efficient feed attractants or stimulants for various fresh water and marine fishes and crustaceans (Heinen, 1980; Kasumyan and Deving, 2003) promoting growth (Deshimaru and Yone, 1978). Feed attractants are usually in the form of small chain peptides, small molecular weight compounds and free amino acids, which are what fish taste buds sense to detect taste (Sutterlin and Sutterlin, 1970). Neutral amino acids with unbranched side chains and a low number of carbon atoms are commonly stimulatory in fish, whereas acidic amino acids elicit low gustatory stimulations, while responses to basic amino acids are dependent on the fish species (Kasumyan and Doving, 2003). Although single amino acid can be quite stimulatory, certain amino acids enhance the electrophysiological response of other amino acids making amino acid combinations more effective for certain fish (Marui and Kiyohara, 1987). Peptides can act as stimulants in fish, but they are far less effective than their component free amino acids (Caprio, 1978). FAAs are important nutritional components which may influence the feeding behaviour and are important energy source for larval fish despite the fact that the FAA used for energy metabolism may be different for different fish species (Claudia, 2004). Certain FAA such as proline, glycine, alanine are important for intracellular isosmotic regulation of penaeid shrimp reared in high

salinities (Schoffeniels, 1970) and also used as additional source of energy to maintain osmotic homeostasis. Dietary addition of free amino acid like arginine could also potentially improve adaptation, growth and survival of shrimp reared in low saline waters.

Fatty acids such as linoleic acid (18:2n-6), linolenic acid (18:3n-3), arachidonic acid (20:4n-6), eicosapentaenoic (20:5n-3) acid and docosahexaenoic acid (22:6n-3) have been deemed essential for growth and development including reproduction of various fishes and crustaceans (Sargent *et al*., 1999; Glencross *et al*., 2002). *Candida* MCCF 101 contained linoleic acid dominating among them followed by stearic and palmitic acids. Linoleic acid, being poly unsaturated fatty acid (PUFA) has commercial applications in aquaculture. Fish feed containing yeast cell supplement with PUFA are required for normal growth and high survival rate of fish larvae (Sagent, 1989; Yamasaki *et al*., 2007) and this requirement can be very well met from *Candida* MCCF 101. Field level experiments of the same marine yeast, conducted by Sajeevan *et al*. (2009a) pointed out that whole cell yeast performed better as immunostimulant in shrimp than the extracted cell wall glucans.

The marine yeast has been regarded as safe and has been showing a beneficial impact on fermentation and biomass production. The result from the studies on *Candida* MCCF 101 has shown that it has all the qualities to make it an ideal feed supplement in aquaculture.

Table.1 Amino acid composition of *Candida* MCCF101

SL.No	Retention time(RT)	Amino Acid	Result
1.	8.44	Aspartic acid	8.67
2.	10.58	Threonine	4.53
3.	11.44	Serine	4.87
4.	13.182	Glutamic acid	14.84
5.	14.68	Proline	3.61
6.	19.67	Glycine	4.51
7.	21.31	Alanine	7.79
8.	_	Cysteine	0.00
9.	27.28	Valine	781
10.	29.43	Methionine	1.66
11.	31.06	Isoleucine	6.27
12.	32.63	Leucine	14.31
13.	35.25	Tyrosine	3.99
14.	37.05	Phenyl alanine	4.88
15.	45.15	Histidine	1.99
16.	46.37	Lysine	4.31
17.	50.78	Arginine	2.56
18.	_	Tryptophan	6.50

Table 2 Fatty acid composition of *Candida* MCCF 101

Sl.No	Retention time (RT)	C-Atoms	Fatty acids	%of composition
1	4.986	12:0	Lauric acid	0.54
2	7.600	14:0	Myristic acid	2.17
3	9.187	15:0	Pentadecanoic acid	0.68
4	10.583	16:1	Palmitoleic acid	6.37
5	10.893	16:0	Palmitic acid	15.66
6	12.294	17:1	Margaroic acid	4.25
7	12.659	17:0	Margaric acid	0.47
8	13.957	18:2	Linoleic acid	22.45
9	14.451	18:0	Stearic acid	18.0

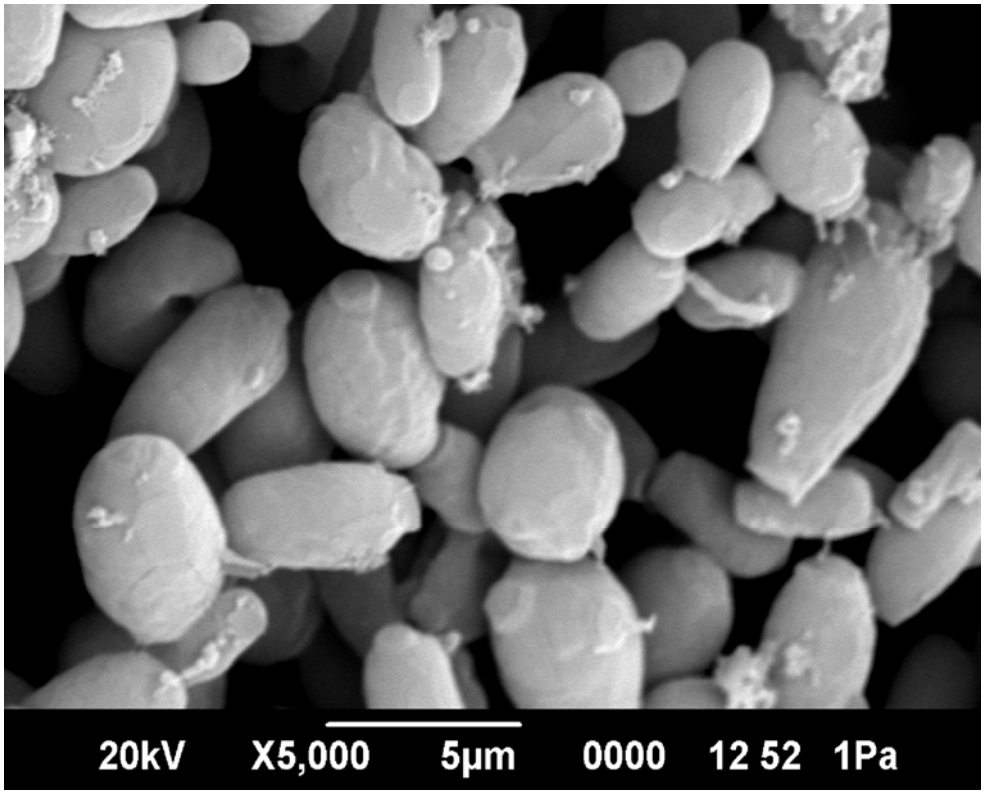

Fig.2 Scanning electron micrograph of *Candida* MCCF 101

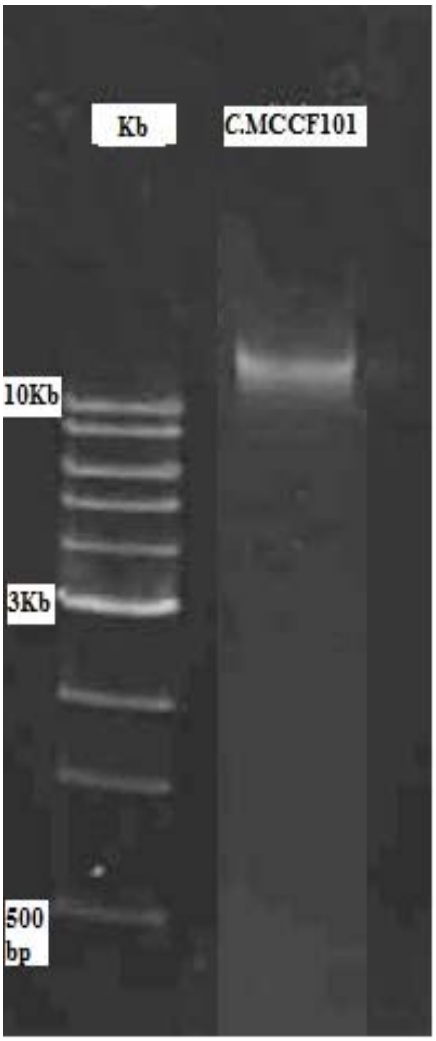

Fig. 3 Genomic DNA of *Candida* MCCF 101

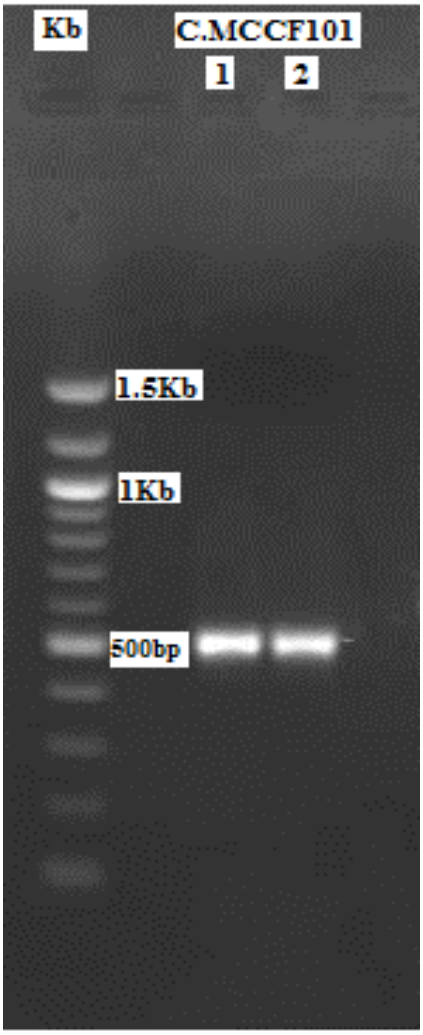

Fig. 4 PCR amplification of ITS region of *Candida* MCCF101

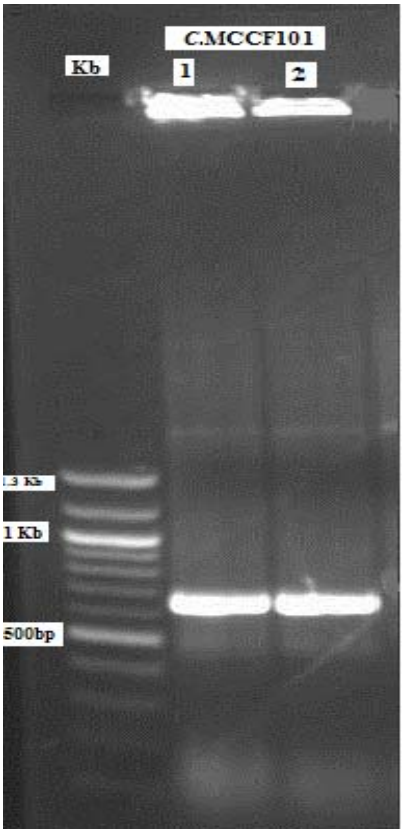

Fig. 5 Colony PCR of clones using T7 and SP6 vector primers with ITS region

```
   1 tccgtaggtg aacctgcgga aggatcatta ctgatttgct taattgcacc acatgtgttt
  61 ttcactggac agctgctttg gcgggggggac tcgtttccgc cgccagaggt cacaactaaa
 121 ccaaactttt tattaccagt caaccatacg ttttaatagt caaaactttc aacaacggat
 181 ctcttggttc tcgcatcgat gaagaacgca gcgaaatgcg atacgtagta tgaattgcag
 241 atattcgtga atcatcgaat ctttgaacgc acattgcgcc ctttggtatt ccaaagggta
 301 tgcctgtttg agcgtcattt ctccctcaag cccgcgggtt tggtgttgag caatacgcca
 361 ggtttgtttg aaagacgtac gtggagacta tattagcgac ttaggttcta ccaaaacgct
 421 tgtgcagtcg gcccaccaaa gcttttctaa cttttgacct caaatcaggt aggactaccc
 481 gctgaactta agcatatcaa taagcggagg a
```

Fig. 6 Partial nucleotide sequence of ITS regions (internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2) of marine yeast *Candida* sp. MCCF 101. The sequence is deposited in GenBank with the Accession No. FJ652052

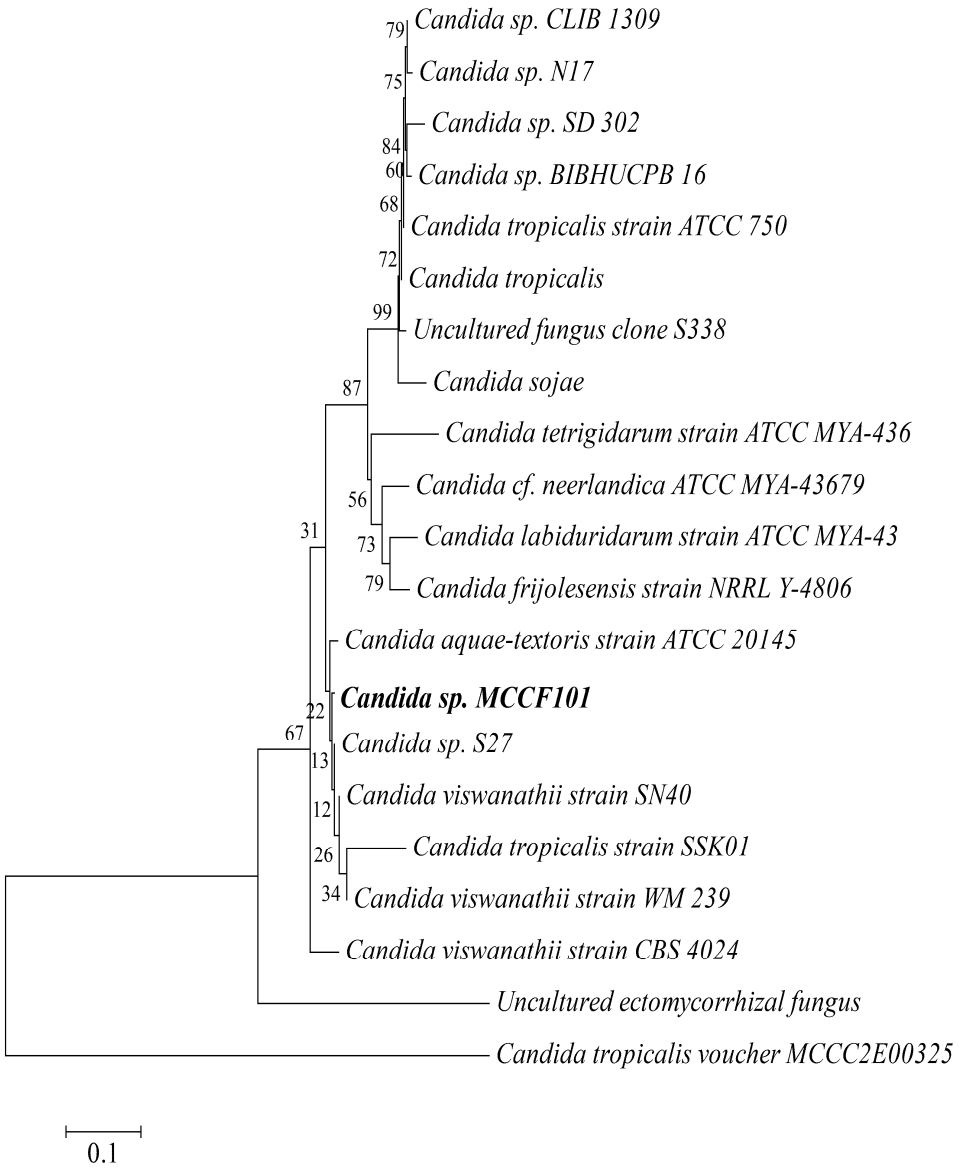

Fig.7 Phylogenetic tree based on the neighbour joining method using the nucleotide sequence of the internal transcribed spacer region of *Candida* MCCF 101 with their most similar matches in GenBank data base. The bootstrap values were fixed at 1000.

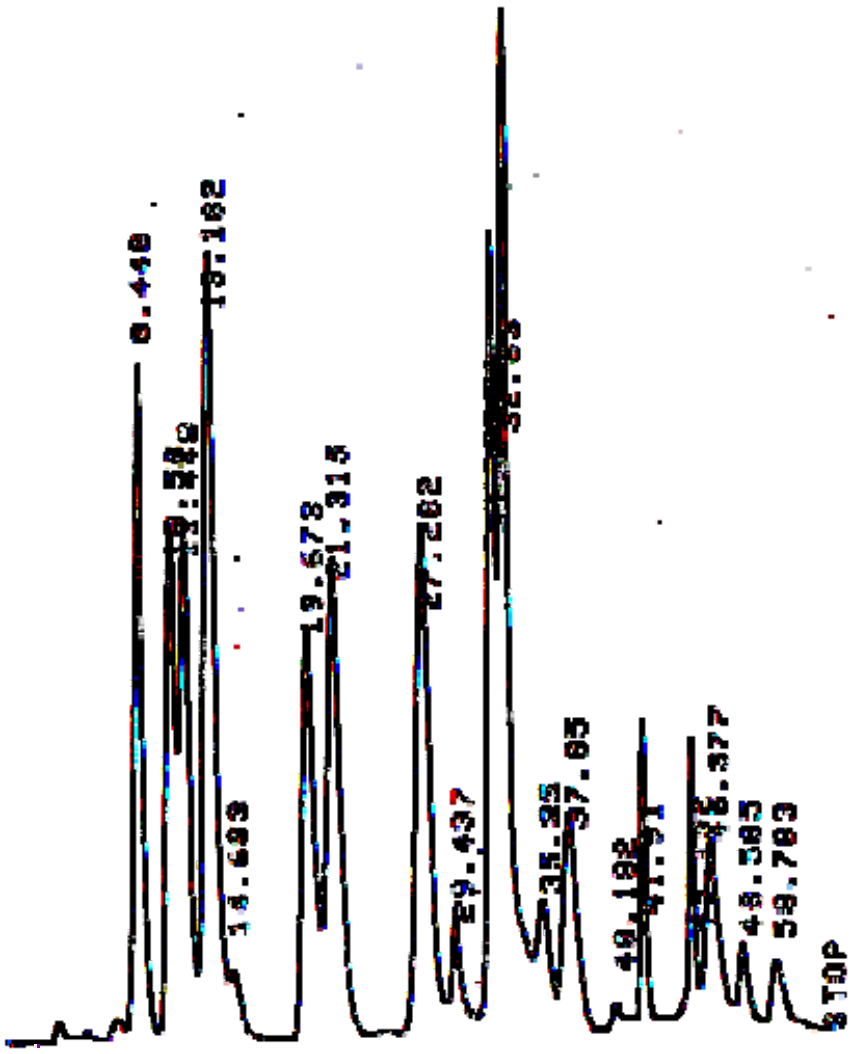

Fig.8 Amino acid chromatogram of *Candida* MCCF 101, each peak representing an amino acid. Peak: 8.448-Aspartic acid, 10.58-Threonine, 11.448-Serine, 13.182-Glutamic acid, 14.683-Proline, 19.673-Glycine, 21.315-Alanine, 27.282-Valine, 29.437-Methionine, 31.812-Isoleucine, 35.25- Tyrosine, 37.05-Phenyl alanine, 45.152-Histidine, 46.377-Lycine and 50.783-Arginine.

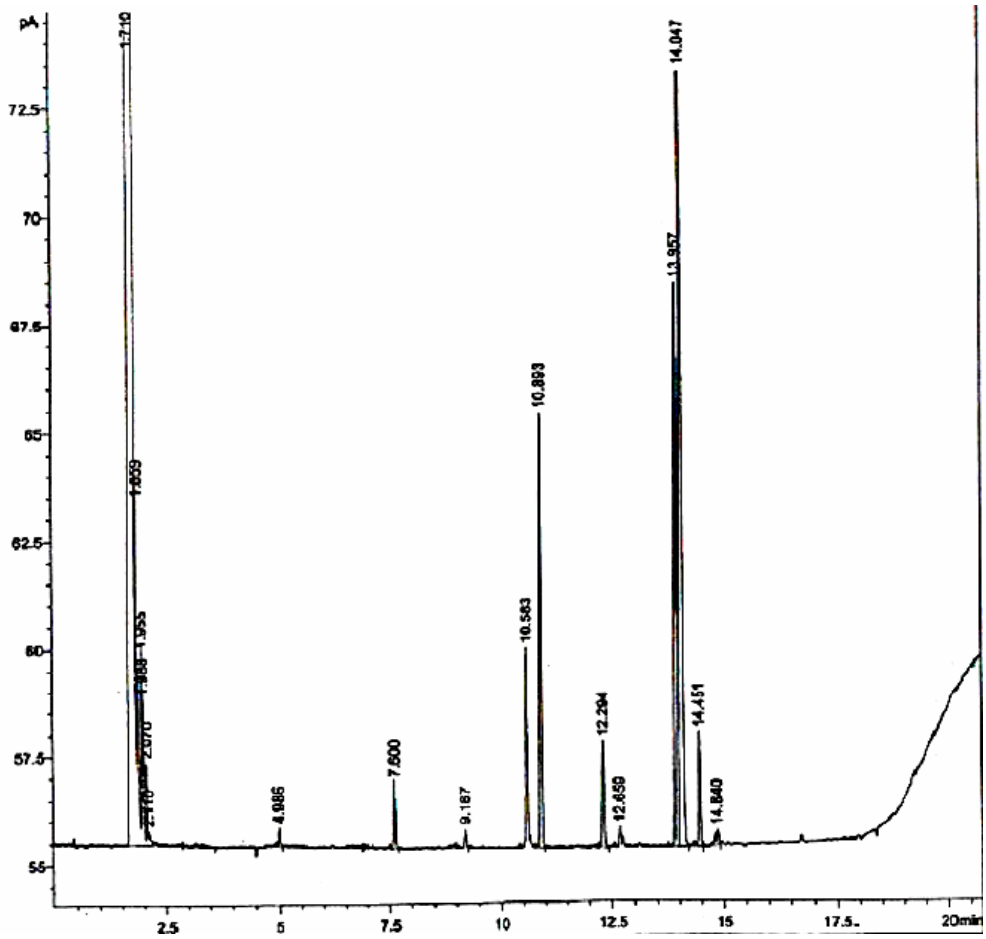

Fig.9 Chromatogram corresponding to the FAME derivative of *Candida* MCCF 101. Each peak corresponds to fatty acids such as Lauric acid (Rt 4.986, C-12:0), Myristic acid (Rt 7. 600, C-14:0), Pentadecanoic acid (Rt 9.187, C-15:0) Palmitoleic acid (Rt 10.583, C-16:1), Margaroic acid (Rt 12.294, C-17:1), Margaric acid (Rt 12.659, C- 17: 0), Linoleic acid (Rt 13.957, C-18:2), Stearic acid (Rt 14.451, C-18:0).

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Chapter 3

OPTIMIZATION OF PHYSICAL PARAMETERS SUCH AS pH, TEMPERATURE AND NaCl CONTENT IN MALT EXTRACT BROTH USING RESPONSE SURFACE METHODOLOGY (RSM) AND GENERATION OF YEAST BIOMASS IN LABORATORY AND PILOT SCALE FERMENTORS

Contents

3.1 Introduction

Traditional industrial attributes of yeasts include their primary roles in many food fermentations such as beers, cider, wines, sake, distilled spirits, bakery products, cheese, sausage and other fermented foods. Newer applications involving yeasts are the production of fuel, ethanol, single cell protein (SCP), feeds and fodder, industrial enzymes, and small molecular weight metabolites. More recently *Komagatella (Pichia) pastoris*, *Saccharomyces cerevisiae*, *Ogataea (Hansenula) polymorph* and certain other yeast species were identified and developed as industrial organisms for the heterologous production of enzymes and proteins, including protein pharmaceuticals. Production of Single Cell Protein (SCP) was another area involving yeasts that contributed greatly to the advancement of present day biotechnology.

A solution to nutrient scarcity, the future of SCP will be heavily dependent on reducing production cost and improving quality by fermentation, downstream processing, and improvement of microbial strains by genetic manipulation and recombinant DNA technology (Omar and Sabry, 1991).

Candida is a genus that includes many species with potential application in biotechnology as single cell protein, probiotic and pharmaceutical applications. *Candida* species include *C. tropicalis, C. maltosa, C. rugosa, and C. utilis* used industrially for the synthesis of SCP. These organisms grow on a variety of substrates and waste materials including oils, plant hydrolysates, apple pomace, sulfite waste liquor and many other substrates and produce biomass.

Usually the production of SCP is carried out in fermentors. This is done by growing selected strains of microorganisms on suitable raw materials in technical cultivation process resulting in large scale biomass production followed by separation process. For economical production and utilization of biomass, selection of appropriate microorganism, optimization of culture conditions, design and operation of fermentors, selection of suitable substrate, time of harvest, post-harvest processing, by-product utilization, waste disposal and recycling, all play major roles. The product should meet the safety guidelines specified for food and the process technology should meet the guidelines specified in environmental protection rules, before it is mass produced and marketed. Product quality meeting the safety guidelines for food and process technology giving thrust to environmental protection are also considered in the production of SCP (Nasseri *et al*., 2011)**.**

3.1.1 Growth of Yeast

The growth of organisms may be seen as the increase of cell materials expressed in terms of mass or cell numbers and results from a highly complicated and coordinated series of enzymatically catalysed biological steps. Growth will be dependent both on the availability and transport of necessary nutrients to cell and subsequent uptake, and on environmental parameters such as salinity, pH, temperature and aeration being optimally maintained. Yeast cells are grown in a series of fermentation bioreactors, which are operated under aerobic conditions to promote growth.

The yeast growth obtained from batch culture is comprised of characteristic lag, exponential and stationary phases. The lag phase represents a period of zero growth and is exhibited when newly inoculated microorganisms experience a change of nutritional status or alterations in physical growth conditions (eg. Temperature, osmolarity etc.).The lag phase reflects the time required for microorganisms to adapt to their new physical and chemical growth environment by synthesizing ribosomes and enzymes needed to establish growth at higher rate. The exponential phase represents a period of logarithmic cell doublings and constant and maximum specific growth rate. Thus an exponentially growing culture will soon exhaust one or more required nutrients and cease to grow. Alternatively waste materials may accumulate to levels that inhibit growth, even if all the nutrients are present in excess. The precise value of maximum specific growth rate of reciprocal time depends on the yeast species and prevailing growth conditions. In the stationary phase the accumulated yeast biomass remains relatively constant and the specific

growth rate returns to zero and after prolonged periods of stationary phase yeast may die and autolyse.

For any study involving microbial biomass a quick and accurate measure of the size and growth kinetics of microbial population is necessary. For this purpose spectrophotometry is the method of choice. The Absorbance of a suspension is defined as follows:

$$\text{Absorbance} = \log (I_o/I)$$

Where I_o is the amount of light entering the specimen, and I is the amount of light emerging from it.

3.1.2 The effect of environmental conditions on biomass production

The specific growth rates of yeast are affected by the environment, both physical and biotic. The principal physical factors affecting microbial growth are pH, temperature and salt concentration. The principal biotic factors are the presence of compounds excreted by other organisms, competition for nutrients, and predation. The starter culture (inoculum) is an important factor for the optimal production of a microorganism. It must be in a healthy, active state thus minimizing the length of the lag phase in the subsequent fermentation, available in sufficiently large volumes to provide an inoculum of optimum size, free of contamination and must retain its product-forming capabilities (Abadias *et al*., 2003). Physical factors such as aeration, agitation, pH and temperature as well as medium constituents may affect the quality and quantity of the desired microorganism (Churchill, 1982). This section, briefly discusses the effects of the physical factors.

3.1.2.1 Physical factors

Most yeast grows well in warm, moist, sugary, acidic and aerobic environment. Biomass production is an important element in fermentation process. Hence, temperature and pH during cell growth are key process variables that must be considered in both the design and cost of the fermentation process (Roebuck *et al*., 1995). For biomass production, high specific growth rate during initial stages of cultivation is of utmost importance for process performance and efficiency. The specific growth rate of yeast depends strictly on the strain, its physiological state and environmental conditions such as pH, temperature and salt concentrations (Kasemets *et al.*, 2007). Usually, these factors also affect the shelf life of food products derived from them (Papouskova and Sychrova, 2007).

3.1.2.1.1 *Salinity*

Collectively, yeasts grow over a wide range of salinity, from essentially zero to saturated $40gL^{-1}$. No single organism can grow over this entire range. Rather, each organism has a range of salinity that it tolerates; outside of this range it cannot grow. Yeasts can be classified into three categories based on its preference to salt content in the medium: Non-halophiles (no salt requirement), halophiles (grows best in media with 15 - $30gL^{-1}$ salinity) and extreme halophiles (grow well in media with 30-$40gL^{-1}$ salinity)

Halophiles have developed unique strategies such as intracellular accumulation of osmolytes and salt-adapted enzymes to withstand salt stress conditions. These properties of halo tolerance are of considerable biotechnological significance (Ventosa and Nieto, 2005). Studies on the

growth of several marine yeasts related to graded NaCl concentrations have revealed a high salt tolerance in members of the genera *Debaryomyces, Pichia,* and *Candida*, isolated from marine fish (Ross and Morris, 1962) or sea water (Norkrans, 1966). Smittle (1977) reported that increasing concentrations of salt shifted the pH optimum for growth of *Zygosaccharomyces bailii* and *Z.acidifaciens* to lower values. In the cultivation medium higher concentration of NaCl caused changes in the composition of extracellular yeast glycoproteins (Breierova, 1997a). Significant changes in the fatty acid composition of the cell lipids and extracellular glycoproteins were observed during various growth phases in the yeast-like species *Dipodascus australiensis* when grown under various salt concentrations (Breierova, 1997b). For most yeast, tolerance of NaCl decreased at the extremes of pH. High NaCl concentrations impose both ionic and hyper-osmotic stress on yeast cells (Blomberg, 2000). The plasma-membrane fluidity in relation to NaCl concentrations in yeasts and yeast-like fungi isolated from either subglacial ice or hypersaline waters was studied by Turk *et al*. (2007).

3.1.2.1.2 *pH*

According to the pH scale the zone of maximal growth allows organisms to be divided in to three groups; neutrophils grow best in the range of pH 6-8; acidophiles grow best at pH values less than about 4 and alkaliphiles grow best at pH values about 9.

Culture pH strongly affects cell growth indirectly by affecting the nutrient availability or directly by action on the cell surfaces. Yeasts and molds can be found in a wide variety of environments due to their capacity to utilize a variety of substrates and their tolerance of low pH values, and low temperatures (Huisin't Veld, 1996). Most yeast prefer a

pH range of 3.0-7.0 (Walker, 1977; Miller, 1979; Deak, 1991). Environmental pH is particularly significant in determining the growth of yeasts in the presence of weak organic acids (Pitt, 1974; Cole and Keenan, 1986) and it may also affect their responses to high concentrations of salt or sugar (Tokuoka, 1993). *Debaryomyces nepalensis*, a halotolerant food spoiling yeast could grow in complex (YEPD) medium at different pH ranging between 3.0 and11.0 in the absence of salt and at pH 3.0–9.0 in the presence of different concentrations of NaCl and KCl (Kumar and Gummadi, 2008). *Zygosaccharomyces rouxii* was able to grow at a wide range of pH values, such as pH 1.8 to 8.0 in the presence of high concentrations of glucose (Tokuoka, 1993) or pH 1.5 to 10.5 in 12% glucose medium (Restaino *et al*., 1983). Du Preez *et al*. (1984) reported that the optimal pH for the growth of *Candida shehatae* was between 3.5- 4.5.

3.1.2.1.3 *Temperature*

Temperature is one of the most important physical parameters which influence yeast growth. Yeasts are grouped according to their thermal domains for growth as psychrophilic, mesophilic and thermophilic. Most laboratory and industrial yeasts generally are mesophilic, which grow best between 20-30^{o}C (Barnett *et al*., 1990). Some psychrophilic yeast grows optimally at temperatures between 12 and 15^{o}C. Higher temperatures in the range of 30-37^{o}C are often required for yeasts that are strictly associated with warm blooded sources. The maximum temperature for growth is relatively constant within a species. Temperature can affect the sensitivity of yeasts to alcohol concentration, growth rate, rate of fermentation, viability, length of lag phase, enzyme and membrane function etc. High

temperature is thought to cause increased fluidity in membranes generally and yeasts respond to this physical change by changing their fatty acid composition (Ohta *et al*., 1988).

Kamel and Kawano (1986) noted that optimum temperature for the growth of *Candida* sp. was 37^{o}C and the growth declined with increase or decrease in temperature. Charoenchai *et al*. (1998) reported that the growth rates of *Saccharomyces cerevisiae*, *Pichia anomala, Kloeckera apiculata, and Torulaspora delbrueckii* increased with temperature up to 25°C. Two marine yeast strains *Debaryomyces hansenii* (Yeast-14) and *Candida austromarina* (Yeast-16) had maximal growth in the temperature range of 20-30^{o}C and 20-25^{o}C respectively (Kang *et al*., 2006). Elevated temperatures usually encountered in several geographical regions may adversely affect alcohol and other industrial fermentation processes (Krouwel and Barber, 1979). Significant reduction in overall biomass and ethanol yield was observed in a thermo tolerant strain of *Kluyveromyces marxianus* (Hughes *et al*., 1984).

3.1.3 Optimization

Process optimization is a topic of central importance in any industrial production. During the development of a fermentation process, the optimization of process variables is given main thrust as it has direct bearing on the economy and feasibility of the process (Kammoun *et al*., 2008). The relevance of this step is such that even small improvements can be decisive for commercial success (Reddy *et al*., 2008). Physical and chemical factors form the first and foremost part of an optimization process. Classical optimization method involves single factor variation, keeping the other factors constant. This method is unsuited for multifactor

optimization. It is time-consuming and is unable to detect the true optimum due to the interactions between the factors (Liu and Tzeng, 1998; Weuster-Botz, 2000). The limitation of such method is avoided using statistical design which has many advantages (Carvalho *et al*., 1997; Li *et al*., 2007; Xiao *et al*., 2007). Statistical experimental design for optimization of fermentation media involves selection of the most significant media components (screening), identification of the significant ranges of the selected variables (narrowing), optimum identification of the variables (optimum search), and experimental verification of the identified optimum (Weuster-Botz, 2000).

3.1.3.1 Shake flask experiment

Shaking bioreactors are well established and useful tool for initial culture experiments, screening purposes and bioprocess development. Screening of wild type strains with specific activities, conventional strain development using mutation and selection, strain development with recombinant techniques, elucidation of metabolic pathways, medium development, establishment of analytical protocols, investigations of basic process conditions like strain stability, inoculum ratio, optimal pH and temperature, total culturing time and the evaluation of fundamental kinetic data are all performed employing extended parallel experiments in shaking flasks (Buchs, 2001). It is crucial to search for the key influencing factors from many related ones. Such work is extremely laborious and time-consuming using the conventional one-factor-at-a-time method (Wasser, 2002; Adinarayana *et al*., 2003). Moreover, it does not guarantee the determination of optimal conditions, and is unable to detect the frequent interactions occurring between two or more factors (McDaniel and Bailey, 1969; Kennedy *et al*., 1994).

3.1.3.2 Response Surface Methodology

Response Surface Methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses (Li *et al*., 2002; Elibol and Ozer, 2002; Lee *et al*., 2003). This statistical technique has been extensively applied in many areas of biotechnology viz. optimization of media (Farrera *et al*., 1998; Elibol, 2004; Chakravarti and Sahai, 2002; Lai *et al*., 2003; Francis *et al*., 2003) cultivation conditions (Sen and Swaminathan, 1997; Vasconcelos *et al*., 2000; Hujanen *et al*., 2001; Triveni *et al*., 2001) and biomass production (Lhomme and Roux, 1991).

The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple factors and their interactions. Also, study of the individual and interactive effects of these factors will be helpful to find the target value. Hence, RSM provides an effective tool for investigating the aspects affecting the desired response if there are many factors and interactions in the experiment. To optimize the process, RSM can be employed to determine a suitable polynomial equation for describing the response surface (Yin *et al*., 2009).

Basically this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model. After this, the levels of the variables giving maximum response can be calculated using the mathematical model (Maddox and Richert, 1977).

The statistical optimization techniques make possible the optimization of state variables such as temperature, pH, agitation, aeration, feeding rates etc together with quantitative variables such as medium components (Harris *et al*., 1990; Bazaraa and Hassan, 1996; Kennedy and Krouse, 1999).

The most extensive applications of RSM are in particular situations where several input variables potentially influence some performance measure or quality characteristic of the process. This performance measure or quality characteristic is called the response. The input variables are sometimes called independent variables. The field of response surface methodology consists of the experimental strategy for exploring the space of the process or independent variables, empirical statistical modeling to develop an appropriate approximating relationship between the yield and the process variables, and optimization methods for finding the values of the process variables that produce desirable values of the response. In many cases, either a first-order or a second order model is used.

For the case of two independent variables, the first-order model in terms of the coded variables is

$$\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2; \quad \text{----------} (1)$$

The form of the first-order model in Equation (1) is sometimes called a main effects model, because it includes only the main effects of the two variables X_1 and X_2. If there is an interaction between these variables, it can be added to the model easily as follows:

$$\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2; \quad \text{----------} (2)$$

This is the first-order model with interaction. Adding the interaction term introduces curvature into the response function.

Often the curvature in the true response surface is strong enough that the first-order model is inadequate. A second-order model will likely be required in these situations. For the case of two variables, the second-order model is

$$\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad \text{----------} (3)$$

Where 'η' is the estimated response, 'β_0' is a constant, 'β_1, β_2, β_3, β_{11}, β_{22}, β_{12}, are the coefficients for each term and 'x_1, x_2 and x_3 are the independent variables.

OR

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\, \beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j, \quad \text{----------} (4)$$

Where Y represents the response variable, β_0 represents the interception coefficients, β_i is the coefficient of linear effect, β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of the interaction effect (Bas and Boyaci, 2007; Jian and Nian-fa, 2007; Venil *et al*., 2009).

The second-order model is widely used in response surface methodology for several reasons:

1) The second-order model is very flexible. It can take on a wide variety of functional forms, so it will often work well as an approximation to the true response surface.

2) It is easy to estimate the parameters (the β's) in the second-order model. The method of least squares can be used for this purpose.

3) There is considerable practical experience indicating that second-order models work well in solving real response surface problems.

The most popular class of second-order designs is the central composite design (CCD) (Myers and Montgomery, 2002). This design can be easily constructed by augmenting the fractional factorial design that is used for estimating the first-order model. The use of CCD allowed determination of levels of various parameters to be carried out with the interrelation between each parameter evolved simultaneously (Shiow-Ling and Wen-Chang, 1997). This method is a well established widely used statistical technique for determining the key factors from a large number of medium components by a small number of experiments and successfully applied in the optimization of medium composition (Khuri and Cornell, 1987; Soni *et al*., 2007). It has three groups of design points:

a) Two-level factorial or fractional design points - all possible combinations of the +1 and -1 levels of the factors (2^n where "n" is the number of factors).

b) Axial points or star points - Axial points (outside the core), often represented by stars, emanate from the center point, with all but one of the factors set to 0. The coded distance of the axial points is represented as a plus or minus alpha ("– α" or "+ α").

c) Center points - points with all levels set to coded level 0 (midpoint). Center points are usually repeated to get an estimate of experimental error.

The variables are coded according to the following equation:

$$\text{Coded value} = \frac{\left[\text{Actual value} - 1/2\left(\text{high level} + \text{low level}\right)\right]}{\left[1/2\left(\text{high level} - \text{low level}\right)\right]}$$

Central Composite Designs are intended to estimate the coefficients of a quadratic model.

Data analysis of RSM also involves the graphical representation of the model equation and determination of optimal operating conditions. The visualization of the predicted model equation can be obtained by the response surface plot or contour plot. The response surface plot is the theoretical three dimensional plot showing the relationship between the response and the independent variables. The two dimensional display of the surface plot is called contour plot and in the contour plot, lines of constant responses are drawn in the plane of the independent variables. The contour plot helps to visualize the shape of a response surface. When the contour plot displays ellipses or circles, the centre of the system refers to a point of maximum or minimum responses. Sometimes, contour plot may display hyperbolic or parabolic system of the contours. In this case, the stationary point is called the saddle point and is neither a maximum nor a minimum point. These plots give useful information about the model fitted but they may not represent the true behaviour of the system (Myers and Montgomery, 1995).

Response surface methodology has been commonly used to optimize the microbial growth, production of enzymes and metabolites (Varela *et al*., 1996; Bankar *et al*., 2008; Valduga *et al*., 2008). Lal *et al*. (2009) determined the best conditions of salt concentration, pH, and temperature for both maximizing and minimizing the growth of halotolerant yeast,

Debaryomyces nepalensis NCYC 3413 using response surface methodology. Response surface modelling was applied to determine the optimum temperature and pH for the biomass production of yeast *Pachysolen tannophilus* in shake flasks (Roebuck *et al*., 1995). The influence of pH and dilution rate on continuous production of xylitol from sugarcane bagasse hemicellulosic hydrolysate by *Candida guilliermondii* using RSM was done by Martinez *et al*. (2003).Optimum conditions of pH, temperature and period of incubation for pectinolytic activity of yeast *Kluyveromyces wickerhamii* isolated from rotting fruits and to assess the effect of these factors by RSM was employed by Moyo *et al*. (2003). Arroyo-Lopez *et al*. (2009) studied the effects of temperature, pH and sugar concentration (50% glucose + 50% fructose) on the growth of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their interspecific hybrid by means of RSM based on a central composite circumscribed design. Li *et al*. (2008) optimized the culture conditions such as concentration of ammonium sulphate, glucose, NaCl and pH for maximizing the production of phytase from the marine yeast *Kodamaea ohmeri* BG3 in an inexpensive oats medium using RSM. Soni *et al*. (2007) studied response surface optimization of the critical media components (mannitol, yeast extract and calcium chloride) and initial pH for carbonyl reductase production by *Candida viswanathii* MTCC 5158 and an enhancement of the growth and enzyme activity 1.3 and 2.3 times respectively were obtained. Using central composite design Sheng *et al*. (2009) found that moisture, inoculation size, wheat bran: rice husk ratio, temperature and pH had great influence on inulinase production by *Cryptococcus aureus* strain G7a in solid state fermentation.

3.1.4 Fermentor and Fermentation process

Fermentors are employed in industrial processes to provide a stable and optimal environment for microorganisms that are cultured for by-products or constituents. It is a specialized container where all the culture conditions that are optimized for the growth and product synthesis can be maintained continuously throughout the process of fermentation under sterile conditions. Fermentors are specially designed to suit the industrial scale fermentation for the production of antibiotics, hormone, vaccines, enzymes and chemicals. It can vary in size from laboratory experimental models of one or two liters capacity to industrial models of several hundred liters capacity. A fermentor is equipped with aerator, which keeps steady supply of oxygen to aerobic process and a stirrer keeps the concentration of the medium uniform. The thermostat is used to regulate temperature, pH detector and other control devices keep the respective parameters constant (Sinclair and Cantero, 1990). Since microbial growth is a time dependent process, it exerts continuous modifications on all process parameters which influence physiology, but most dramatically, over substrate concentration. Therefore a process technology which maintains appropriate growth conditions for a prolonged period of time must be implemented for obtaining high yield and productivity values (Nasseri *et al*., 2011).

The first and foremost requisite to any fermentation process is the microorganism that produces the product of economic importance. The starter culture of this organism must have come about after a series of selection and evaluation processes. Sometimes the biomass of the organism must be of significance. If that is the case, the optimization process must concentrate on the production of maximum biomass. Sometimes an

enzyme, a protein or some other by-product must be of significance. Then the optimization process will look at maximizing the production process of the particular ingredient maintaining its desired quality and quantity.

The fermentation process requires axenic culture of the chosen organism in the correct physiological state.

3.2 Materials & Methods

3.2.1 Yeast strain

The yeast strain was sourced as described under section 2.2.1. The yeast was cultured in malt extract medium in sea water having salinity 20parts per thousand and composed of malt extract, 17gL^{-1}, mycological peptone, 3gL^{-1}, agar, 20gL^{-1}.

3.2.2 Inoculum Density

To determine the inoculum density to be used for the optimization experiments, malt extract broth was inoculated with yeast cell suspension of varying cell count expressed in terms of absorbance 540nm, such as 0.025 (1.39×10^5 cfu mL^{-1}), 0.05 (2.79×10^5 cfu mL^{-1}), 0.10 (5.57×10^5 cfu mL^{-1}), 0.15 (8.44×10^5 cfu mL^{-1}), 0.2(11.14×10^5 cfu mL^{-1}) and incubated at 25.0 ±1^oC for 96 hrs on a rotary shaker at 120 rpm. The yeast biomass versus absorbance was measured at fixed time intervals and the optimum inoculum size determined.

3.2.3 Preparation of inoculum

A young (24hrs) lawn culture of the yeast was harvested in to sterile seawater of salinity 20gL^{-1}. Optical density at 540nm was adjusted to 0.1

absorbance which gave a cell count of (5.57×10^5cfu mL^{-1}) using sterile sea water. This formed inoculum for further experiments.

3.2.4 Optimization of physical factors for yeast biomass production

3.2.4.1 Shake flask experiment

The optimization of growth conditions was carried out in Erlenmeyer flasks (250ml) with 50ml malt extract broth.

3.2.4.1.1 *Optimization of NaCl content*

Malt extract broth was prepared in 50 ml aliquots at different salinities such as 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 by addition of NaCl (gL^{-1}) to distilled water. The flasks were inoculated with 0.1ml cell suspension having an absorbance of 0.1 at Abs_{540} nm and incubated at 28±1°C for 72 hrs on a rotary shaker at 120 rpm. Growth was measured as absorbance at 540 nm using a UV- Vis spectrophotometer (UV-1601, Shimadzu Corporation, Tokyo, Japan).

3.2.4.1.2 *Optimization of pH*

Malt extract broth was prepared in 50 ml aliquots in saline water (20 gL^{-1}) optimized from the previous experiment. pH was adjusted to 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5 using 1N HCl and 1N NaOH, inoculated and incubated, and growth measured as detailed earlier.

3.2.4.1.3 *Optimization of Temperature*

Malt extract broth prepared in 50 ml aliquots in saline water (20 gL^{-1}) having pH adjusted to 4.5 was inoculated with yeast suspension as described previously. The flasks were incubated at different temperatures such as 20, 25, 30, 35, and 40ºC in a temperature controlled rotary shaker

(Orbitek - Scigenics Biotech. (Pvt) Ltd, Chennai, India) at 120 rpm.Growth was measured using spectrophotometer at Abs $_{540nm}$.

3.2.4.1.4 *Analysis of growth at different time intervals*

Samples were aseptically withdrawn from the flasks at 24 hours intervals. Cells were separated by centrifugation at 7500 x g for 10 min at 4^oC. The pellets were repeatedly washed in sterile saline ($5gL^{-1}$ NaCl), re-suspended in fresh saline and absorbance measured at 540nm in a UV-Vis spectrophotometer and converted to dry cell mass using a standard curve constructed as described by Guerra and Pastrana (2002).

3.2.4.2 Experimental Design

3.2.4.2.1 *First step optimization*

a) One-dimensional screening

One dimensional screening was done initially to find out the range that has to be used for further optimization experiment. The minimum and maximum ranges of selected variables such as salinity, pH and temperature of the growth medium were determined by varying one factor at a time, keeping others constant. The range and the levels of variables investigated in this study are given in Table 1. An appropriate range for each factor was determined for response surface methodology (RSM). The design contained 20 experiments, 15 different combinations with 6 replications at the centre point (i.e. 2^3 = 8 factorial points, 2 x3= 6 axial points and 1 center point) to locate the true optimum concentrations of the three variables.

3.2.4.2.2 *Second step optimization by RSM*

Response surface methodology (RSM) is an empirical statistical modeling technique employed for multiple regression analysis using

quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously (Rao *et al*., 2000). Here RSM was used to determine the optimum response of *Candida* MCCF 101 for the production of single cell protein. The most popular response surface methodology design is full factorial central composite design (CCD), and has been used here to predict the maximum biomass at different combinations of sodium chloride concentration, pH, and temperature. A 2^3 full factorial central composite design was generated in order to study the effect of the physical factors such as salinity, pH, and temperature as well as to obtain the combination of values that optimize the response within the region of three dimensional observation spaces, which allow designing a minimal number of experiments. The statistical analysis of the results was performed with the aid of Design Expert version 6.0.9 statistical software (Stat-Ease Inc, Minneapolis, MN, USA).The growth was analyzed using the analysis of variance (ANOVA) combined with the Fischer test to evaluate whether a given term has a significant effect ($P \leq 0.05$). The optimum levels of the variables were obtained by graphical and numerical analysis using Design Expert program.

Final equation in terms of coded three factors was:

$$Y = b0 + b1.X1 + b2.X2 + b3.X3 + b12.X1X2 + b23.X2X3 + b13.X1.X3 + b11.X1^2 + b22.X2^2 + b33.X3^2 \quad (5)$$

3.2.4.2.3 ***Experimental verification of the identified optima from the model***

Experiments were repeated at the optimum concentration of sodium chloride, pH and temperature obtained from the model equations for biomass of *Candida* MCCF 101.

3.2.5 Fermentation Experiments:

3.2.5.1 Biomass production in lab scale fermentor:

The fermentation was carried out in a 5L fermentor (Biostat- B-lite bench top fermentor, Sartorious, Germany) containing 3L malt extract broth (Fig.1). The vessel was sterilized at 121^{o}C for 60 min. It was inoculated with 0.1 % (v/v) yeast suspension. The optimized experimental conditions using RSM were adopted (NaCl 0 gL^{-1}, pH 6.51 and temperature 26.3°C) and samples were withdrawn every 24 hrs to estimate the growth of the yeast. In addition, aeration 2.5 LPM (Litre Per Minute) and agitation 300 rpm (revolution per minute) were provided and incubated at 26.3±1^{o}C for 120 hrs.

3.2.5.2 Biomass production in a pilot scale fermentor

Pilot scale fermentation was carried out in a 100L insitu sterilizable fermentor (Scigenics PVT Ltd., Chennai, India) containing 75 L culture medium (Fig. 2) maintaining the same conditions of salt content, pH and temperature as in the case of laboratory scale fermentation. Cell biomass was harvested at the exponential phase and cells were separated by centrifugation at 7500 x g for 10min at 4^{o}C in heavy duty centrifuge (Fig.3) for preparing single cell protein. Absorbance Vs dry biomass was determined following Guerra and Pastrana, (2002).

3.3 Results

3.3.1 Effect of inoculum on biomass production

Optimization of inoculum determined by analysis of variance (ANOVA), which showed that there were no significant differences (P value=0.96033) in inoculum size from 0.025 (1.39x10^{5}cfu mL^{-1}) to 0.2

($11.4x10^5$cfu mL^{-1}). Hence the cell suspension having absorbance 0.1 at Abs_{540nm} ($5.57x10^5$cfu mL^{-1}) (Fig.4) was chosen as the inoculum size for further studies.

3.3.2 Optimization of yeast biomass production

3.3.2.1 One-dimensional screening

Under one dimensional screening to find out the optimum range of each parameter such as NaCl content, pH, and temperature, regression analysis was performed (Table 2) and wherever found significant, the corresponding range was accepted for further analysis. Accordingly, NaCl content 0-50gL^{-1} (Fig.5a), pH 3.5-7.5 (Fig.5b), and temperature 20-35ºC (Fig.5c) were the optimum ranges used for the application of RSM. Absorbance of 1 of yeast cells in suspension (wet weight) corresponds to 0.4669gL^{-1} dry weight.

3.3.2.2 Central composite design and response surface analysis

The influence of NaCl content, pH and temperature on biomass was investigated using RSM. The level of the variables for the CCD experiments was selected according to the results obtained from previous experiments. The design matrix and the corresponding experimental data are shown in Table 3. Experimental results of the CCD were fitted with the second order polynomial equation (3)

$$\text{Biomass (Y)} = -23.195 - 0.067A + 2.956B + 1.386C - 0.0003A^2 - 0.245B^2 - 0.027C^2 - 0.008AB + 0.004AC + 0.008BC \quad \text{----------(6)}$$

(Where 'Y' is the predicted biomass and A, B and C are the coded values of NaCl content, pH and temperature respectively).

Statistical testing of the method was done by standard analysis of variance (ANOVA). The analysis of variance of the quadratic regression model demonstrated that the model was highly significant (P>0.0001) for biomass production. The model F-value 25.07 for biomass also implied that the model was significant.

The 'lack-of-fit' value (0.0722) was insignificant for biomass. Goodness of fit of the model was checked by coefficient of determination (R^2) which could also be expressed in percentage and was interpreted as the percentage variability in the response in the given model. R^2 was 0.9576 in the case of biomass production. As per the model, the sample variation of 95.76% was attributed to the independent variables and the model did not explain 4.24% of the total variation. A higher value of the correlation coefficient (r = 0.9786) indicated that an excellent correlation existed between experimental and predicted values.

The purpose of statistical analysis was to determine which of the experimental factors generated large signals in comparison to the noise. Adequate precision measures the signal to noise ratio and a ratio greater than four was desirable (Wang and Lu, 2004). Adequate precision obtained in this experiment was 13.323. The value of "Pred R^2" of 0.7283 was in reasonable agreement with the "Adj R^2" of 0.9194, which indicated a good agreement between the experiment and the predicted values for biomass production.

The P values were used to assess the significance of each coefficient and the pattern of interaction between the coefficients for biomass production. The smaller the P value, the more significant the corresponding

coefficient (Rao *et al.*, 2000). Linear coefficient B, quadratic coefficients B^2 and C^2 and interaction coefficients AB and AC are significant model terms. As it was a hierarchical model the insignificant coefficients were not omitted from the equation (6) (Wang and Lu, 2004)

The response surface methodology and contour plot to estimate biomass concentration over independent variables such as NaCl content and pH are shown in Fig. 6 (a), interaction of NaCl content and temperature in Fig. 6(b) and pH and temperature in Fig.6(c). The optimum values given for maximum biomass production were NaCl content 0ppt, pH 6.51 and temperature 26.3ºC. In this set-up, the model predicted a maximum biomass concentration of $4.63gL^{-1}$.

3.3.2.3 Validation of the model

The validation was carried out by shake flask experiment under optimum conditions of physical parameters predicted by the model. The experimental value for biomass production ($4.53\pm0.005gL^{-1}$) was closer to the predicted value ($4.63gL^{-1}$) validating the model. By using RSM for optimization of physical parameters, the yield was increased by 11.85% ($4.05 \pm 0.24gL^{-1}$ to $4.53 \pm 0.005gL^{-1}$) compared to biomass production under the laboratory conditions prior to optimization by RSM.

3.3.3 Biomass production in fermentors

This was carried out in 5L lab scale and 100L pilot scale fermentors. The maximum biomass production attained was $7.4gL^{-1}$ (Fig.7a) in 5L lab scale fermentor and $11.54gL^{-1}$ (Fig.7b) in 100L pilot scale fermentor subsequent to 72 hrs of fermentation and cells separated by centrifugation for preparing single cell protein (Fig.8).

3.4 Discussion

Physical parameters viz., salinity (NaCl concentration), pH and temperature are the variables addressed in preliminary experiments considering the design and economy of fermentation process (Pitt, 1974; Cole and Keenan, 1986; Hagler and Ahearn, 1987; Rose, 1987; Roebuck *et al*., 1995; Sorensen and Jakobsen, 1997; Walker, 1998; Martinez *et al*., 2003; Li *et al*., 2008; Lal *et al*., 2009; Sheng *et al*., 2009). Kang *et al*. (2006) reported that the cell growth of two marine yeast strains *Debaryomyces hansenii* (Yeast-14) and *Candida austromarina* (Yeast-16) varied depending on the gradients of NaCl concentration, pH and temperature. Environmental pH is particularly significant in determining the growth of yeasts (Pitt, 1974; Cole and Keenan, 1986). Yeasts are known to grow over a broad pH range from 2 to 9 (Hagler and Ahearn, 1987). Anas and Singh (2003) reported that the yeast *Acremonium diospyri* preferred pH 4 for higher cell yield.

The preliminary experiment was one dimensional screening (initial screening experiment) of growth conditions in order to find the significant range of physical factors affecting the biomass production.

The yeast *Candida* MCCF 101 was capable of growing in a range of 0 -100gL^{-1} concentration of NaCl in shake flask experiments. The growth exponentially increased with the concentration of NaCl from 0 - 40gL^{-1} but the production slightly decreased at concentrations 50-80gL^{-1} NaCl and noticeably decreased from 80-100gL^{-1}. Maximum biomass was obtained at the concentration of 20gL^{-1}. In the case of pH it was observed that the biomass production was possible within the range of pH 3.5-7.5 and the maximum was obtained in pH 4.5. *Candida* MCCF 101 grew well at

temperature in the range 20-35^{o}C and maximum production was observed at 25^{o}C. This screening experiment considered the significance of one factor at a time and did not take into account, the interaction of physical parameters as a whole in the biomass production.

A regression analysis was undertaken to select the optimum range from one-dimensional screening. When the results were found significant the range and levels were subjected to RSM.

The response surface methodology allowed a rapid screening of the important physical parameters that influenced biomass production of *Candida* MCCF101 and it developed a polynomial model to optimize the bioprocess. The results clearly showed that pH, temperature and NaCl content had important roles in biomass production of *Candida* MCCF 101.

The quadratic regression model generated was found highly significant to maximize biomass production. The goodness of fit of the model was checked by coefficient of determination (R^2) which was very high for the biomass production. Adequate precision was obtained for biomass production (13.323) which measured the signal to noise ratio and a ratio greater than 4 was desirable (Wang and Lu, 2004).

The graphical representation of 3D response surface and 2D contour plots (Elibol, 2004) provided a method to visualize the relation between the response and experimental levels of each variable in order to deduce the optimum conditions (Wang and Lu, 2004). When the shape of contour plot is circular, the interactions between the variables are negligible and if it is elliptical the interaction between the variables are significant (Wang and Lu, 2004).

In this case linear and quadratic effects of pH were significant playing an important role in biomass production. In the interaction of salinity and pH, the interaction effect was found significant as elliptical shape was obtained in 2D contour plot. In the case of temperature, linear effect was not significant but quadratic effect was significant. The interaction effect between temperature and salinity were significant, demonstrated by the elliptical shape in 2D contour plot. The interaction effect between temperature and pH was not significant because circular shape was obtained in 2D contour plot. Besides, the linear and quadratic effects of NaCl content were not significant but the interactions between both salinity and pH and salinity and temperature were significant. The model implied that all the three factors such as salinity, pH and temperature had significant effect on biomass production.

Thus, after the optimization using response surface methodology, the optimal parameters for the mass production of marine yeast MCCF 101 were salinity 0.0 $^{0}/_{00}$, temperature 26.3^{o}C and pH 6.51.

Kriss (1963) reported that the majority of yeast in the sea are not accidental forms but species adapted to the life under marine conditions. During one dimensional screening of the parameters such as NaCl content, temperature and pH for the yeast *Candida* MCCF 101, NaCl content of 20gL^{-1} was found to be the optimum. However, under central composite design, NaCl was found not required for optimum growth. Considering the above it is hypothesized that the yeast *Candida* MCCF 101 is a marine isolate rather than autochthonous marine yeast. The experimental verification of the optimum values in the shake-flask experiment demonstrated an increased biomass production by 11.85% under 0.0gL^{-1} salinity.

Candida MCCF 101 cells exhibited higher multiplication rates in the growth medium and the cell density was high. In this study growth of *Candida* MCCF101 under optimized culture conditions derived from response surface methodology produced a cell density of $8.10gL^{-1}$ in 5L lab scale fermentor and $11.54gL^{-1}$ in 100L pilot scale fermentor after 72 hrs of fermentation. Zhenming *et al.* (2006) suggested that the fermentation period of yeast was very short when compared to algae.

Most yeast cells are characterized by flocculation and as the cell size of yeast is much bigger than that of bacteria it is comparatively easy to harvest from liquid culture. In the case of MCCF 101, the diameter of cell was 5µm and the cells showed flocculation and settled to the bottom when kept idle. In an industrial scale, it could be centrifuged completely at 3500 x g in 5 minutes.

Another important attribute that makes it a suitable candidate for SCP production is its optimum temperature for growth, $26.3^{o}C$, the temperature obtained after RSM is the normal room temperature in tropical countries. Therefore, this fermentation process can be carried out without special equipment for controlling temperature, making the process economically viable.

The $0.0gL^{-1}$ salt concentration obtained after RSM also has its advantages. Besides reducing cost of media and labour, it also reduces corrosion and scaling of the equipment which would have happened if a saline media had been used.

Table 1 Experimental range and levels of the independent variables

Variables	Code	Range studied	Range and levels		
			-1	0	1
Salinity (gL^{-1})	A	0-50	0	25	50
pH	B	3-7.5	3.5	5.5	7.5
Temperature(ºC)	C	20-35	20	27.5	35

Table 2 Factor levels used for regression analysis and their significance in the screening experiment of *Candida* MCCF 101

Salinity (gL^{1})	Significance	pH	Significance	Temperature(ºC)	Significance
0	0.023	3.5	0.036	20	0.069
10	0.045	4.5	0.033	25	0.033
20	0.031	5.5	0.034	30	0.02
30	0.05	6.5	0.015	35	0.031
40	0.029	7.5	0.022		
50	0.047				

Table 3 Central Composite Design (CCD) matrixes of the three variables along with the experimental and predicted values of *Candida* MCCF 101.

Expt.No	NaCl (gL^1)	pH	Temperature(°C)	Biomass ($g\ L^{-1}$)	
				Experimental	Predicted
1	0	3.5	20	1.36	1.5
2	50	3.5	20	0.51	0.31
3	0	7.5	20	2.68	3.26
4	50	7.5	20	0.2	0.48
5	0	3.5	35	0.28	0.079
6	50	3.5	35	2.59	2.1
7	0	7.5	35	2.08	2.37
8	50	7.5	35	2.85	2.8
9	-17.04	5.5	27.5	4.56	4.12
10	67.04	5.5	27.5	3.17	3.48
11	25	2.14	27.5	0.024	0.51
12	25	8.86	27.5	3.19	2.58
13	25	5.5	14.89	0.002	-0.43
14	25	5.5	40.11	0.003	0.32
15	25	5.5	27.5	4.5	4.31
16	25	5.5	27.5	4.01	4.31
17	25	5.5	27.5	4.03	4.31
18	25	5.5	27.5	4.68	4.31
19	25	5.5	27.5	4.6	4.31
20	25	5.5	27.5	4.05	4.31

Fig.1 Biomass production in a 5L fermentor containing 3L malt extract broth

Fig.2 Pilot scale biomass generation in a 100L in situ sterilizable fermentor containing 75L using malt extract broth

Fig.3 Yeast cells separated by centrifugation in heavy duty centrifuge

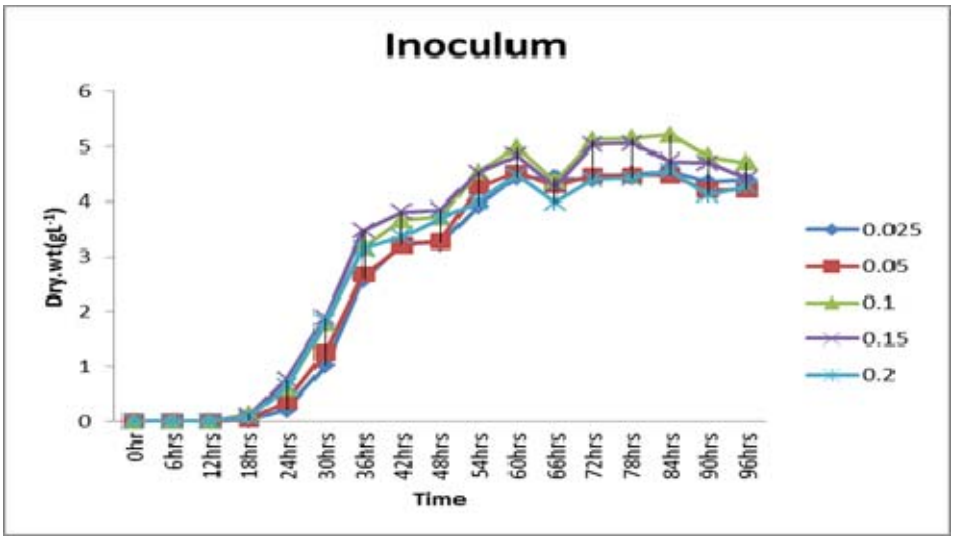
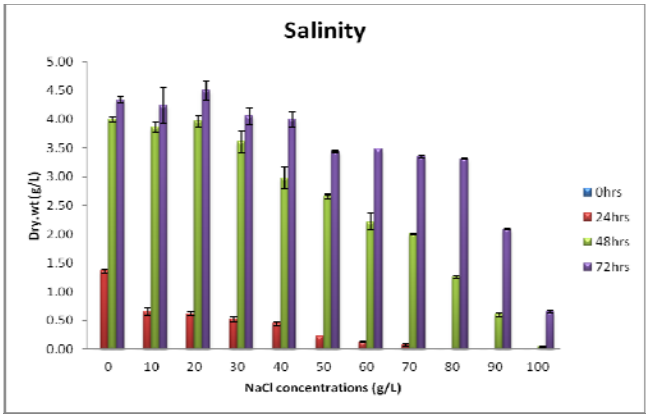
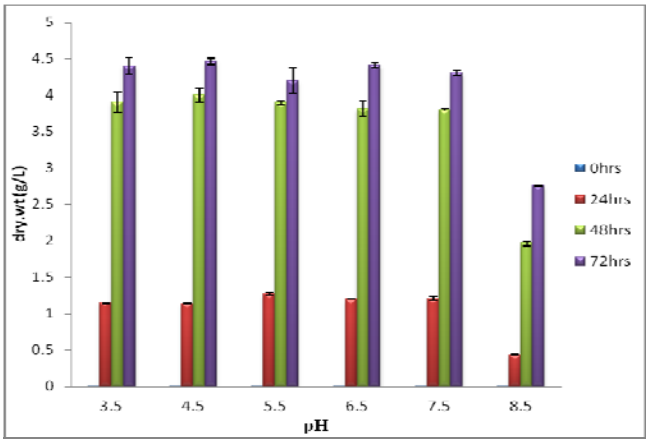


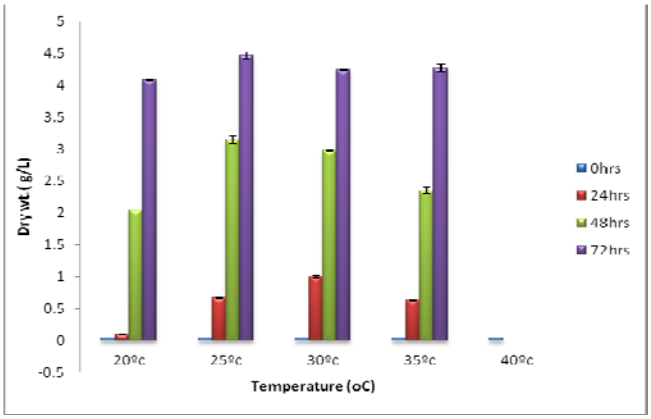
Fig.4 Effective of inoculum on biomass production



(a)

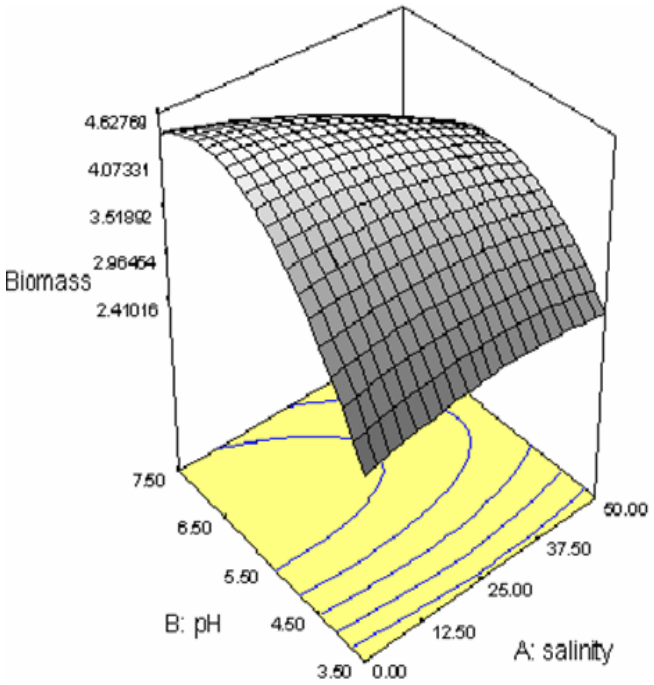


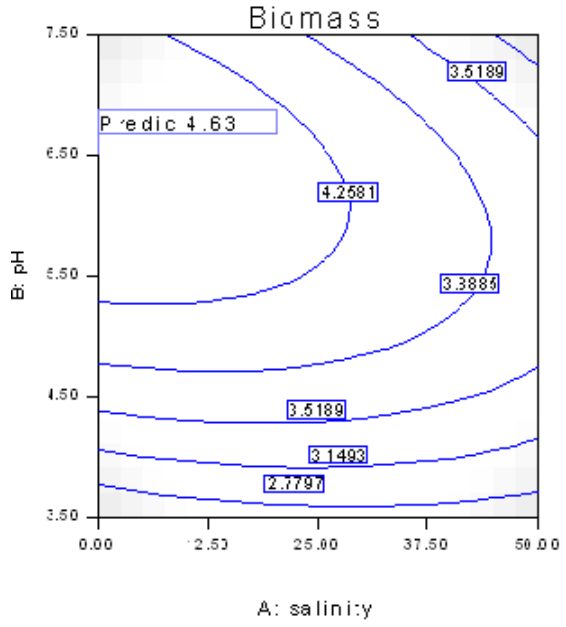
(b)



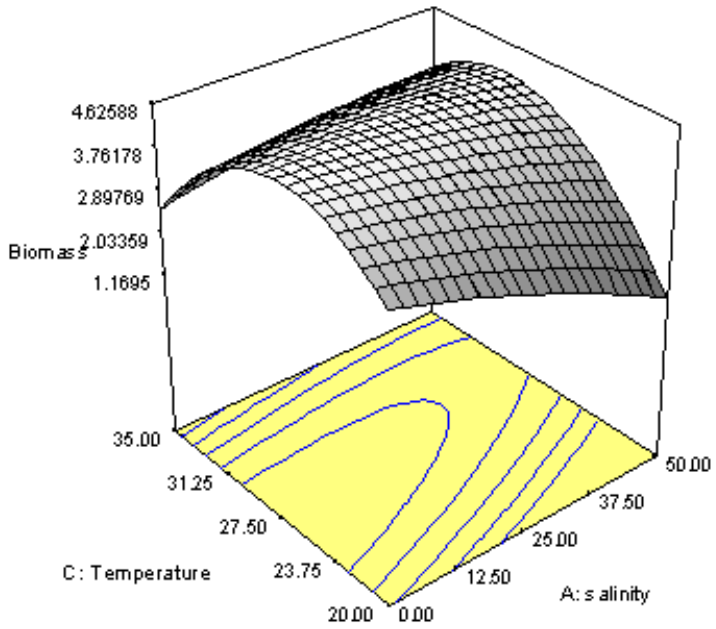
(c)

Fig.5 (a-c) One dimensional screening of physical factors such as NaCl, pH and temperature affecting biomass production


4.62769
4.07331
3.51892
2.96454
2.41016
Biomass
7.50
6.50
5.50
4.50
3.50
B: pH
0.00
12.50
25.00
37.50
50.00
A: salinity


Biomass
Predic 4.63
3.5189
4.2581
3.3883
3.5189
3.1493
2.7797
7.50
6.50
5.50
4.50
3.50
B: pH
0.00
12.50
25.00
37.50
50.00
A: salinity

(a)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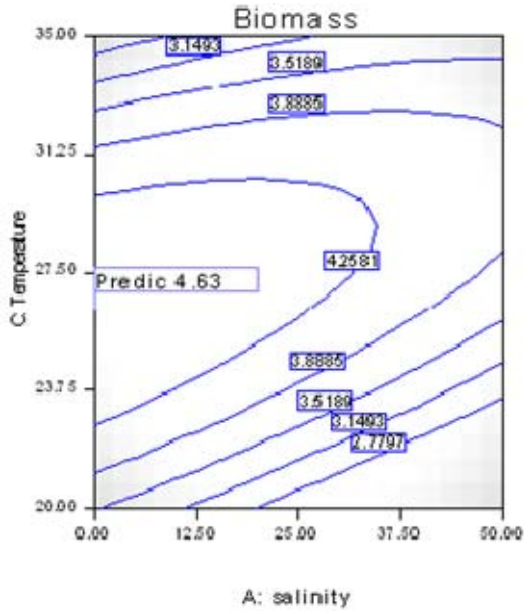
3.76178
2.89769
2.03359
1.1695
Biomass
35.00
31.25
27.50
23.75
20.00
C: Temperature
0.00
12.50
25.00
37.50
50.00
A: salinity


Biomass
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31.25
27.50
23.75
20.00
C: Temperature
3.1493
3.5189
3.8885
4.2581
Predic 4.63
3.8885
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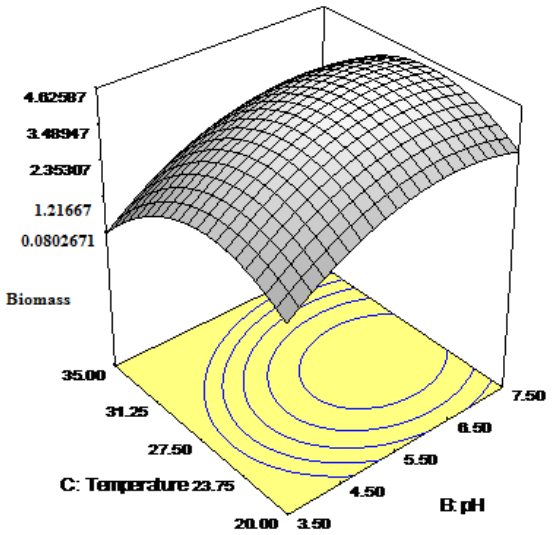
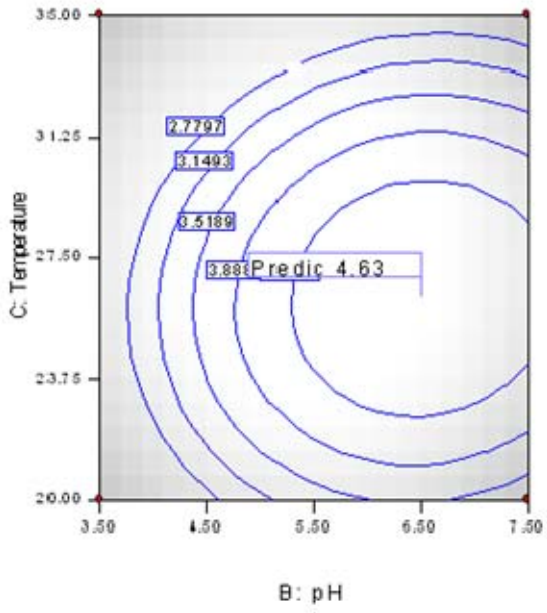
3.1493
2.7797
0.00
12.50
25.00
37.50
50.00
A: salinity

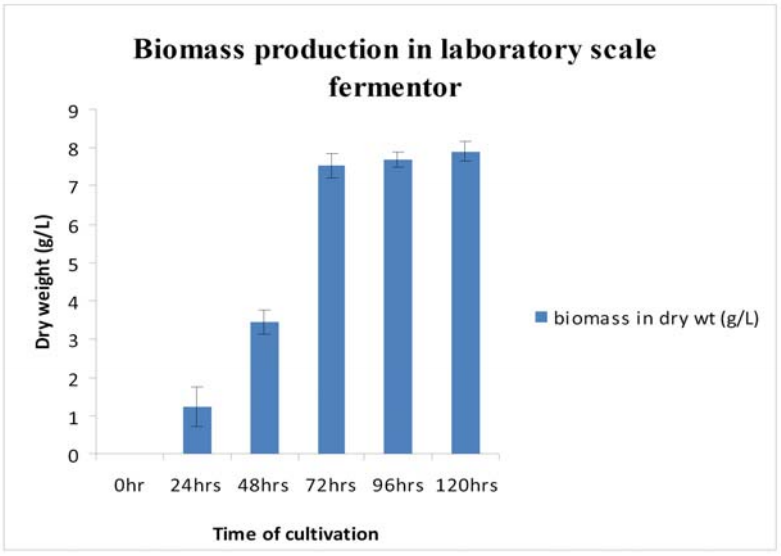
(b)



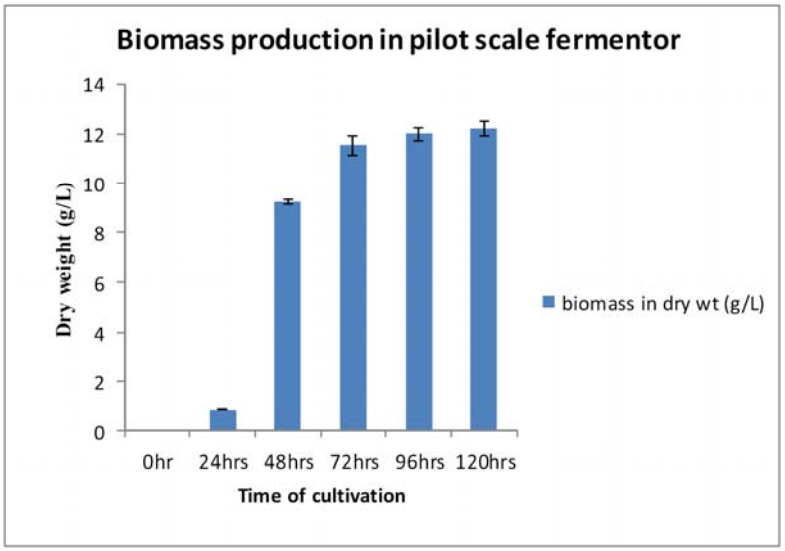


(c)

Fig.6 (a-c) Contour plots and three dimensional plots for the optimization of biomass production. Contour plot and 3D plot respectively showing the interaction between (a) NaCl content and pH (b) NaCl content and temperature (c) pH and temperature.



(a)



(b)

Fig.7(a-b) Biomass production in laboratory scale fermentor (a) and pilot scale fermentor (b).

Fig.8 Packed single cell protein

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Chapter 4

OPTIMIZATION AND SCALE-UP OF YEAST BIOMASS PRODUCTION IN MINERAL BASED MEDIUM AND COMPARISON WITH THAT IN A COMMERCIAL FERMENTATION MEDIUM

Contents

4.1 Introduction

In recent years increasing attention has been given to the conversion of industrial by-products into valuable products such as Single Cell Protein (SCP). SCP production is an efficient way of converting any waste carbohydrate into protein-rich feedstuff. The recovery of value added products and the simultaneous reduction of the organic load of the waste are the chief advantages of such processes. Generation of protein rich biomass from a waste that would have added to the cost of disposal and thereby earning carbon credits is always an attraction in industrial sector (Skogman, 1976; Nigam and Kakati, 2002).

From time immemorial mushrooms have been cultured on waste material by solid state fermentation and consumed by humans. Currently the statistics of mushroom production is so vast and the substrates used vary from feedstock and grass clippings to household garbage.

Yeasts are probably the most widely accepted and used microorganisms for single cell protein. These include strains of *Candida utilis, C. arborea, C. pulcherrima and Saccharomyces cerevisiae* (Dhanasekaran *et al.,* 2011). Yeast single cell proteins are playing a greater role in the evolution of aquaculture diets. Yeasts cell wall containing β- 1, 3 glucan as a potent immunostimulant, has significant augmenting effects on the host defense systems. Yeasts are adapted to grow under stirred fermentation which requires less time for attaining maximum growth and bulk, industrial scale operations can be carried out economically (Hanson, 2008).

4.1.1 Nutritional requirements of yeast

Yeasts are extremely diverse in their nutritional requirement and this diversity will now be considered in terms of the chemical source of the yeast nutrients. Yeast require major elemental building blocks (carbon, hydrogen, nitrogen, oxygen, phosphorus and sulphur) of macromolecules (protein, polysaccharides, nucleic acids lipids) together with bulk inorganic ions (magnesium, potassium) and trace elements (calcium, zinc, copper, and manganese) which play a variety of structural and functional role in yeast cells. A supplement of mineral nutrients is required for the production of yeast biomass. The growth of yeast is concerned with how cells transport and assimilate nutrients and then integrate various functions in the cell in order to increase in mass and eventually divide. Most yeasts grow quite well in simple nutritional media. The culture medium supplies nutrients for growth, energy, building of cell substance, and biosynthesis of products (Walker, 1998).

4.1.1.1 Carbon

Yeasts are chemoorganotrophs they obtain energy from the oxidation of organic compounds. These compounds are mostly a variety of sugars such as dextrose, galactose, maltose, sucrose, fructose, lactose, xylose and raffinose. Some are taken up passively by the cell in an intact form, some are hydrolysed outside the cell and breakdown products are absorbed and others are actively transported across the cell membrane and hydrolysed in the cytosol of the cell. Different types of carbon sources can be assimilated by various yeasts with varying degree of effectiveness. The carbon substrate has a dual role, both in biosynthesis and energy generation with carbohydrates being the usual carbon source for microbial fermentation process (Casida, 1968; Stanbury *et al.*, 1995)

4.1.1.2 Hydrogen

Elemental hydrogen is present in yeast cell macromolecules and is obtained from carbohydrate and other sources. Hydrogen ions are very important in yeast physiology since both extracellular and intracellular pH has a dramatic influence on growth and metabolism of yeast cells (Walker, 1998).

4.1.1.3 Nitrogen

Yeasts are capable of utilizing a range of different inorganic and organic sources of nitrogen for incorporation into the structural and functional nitrogenous components of the cell, such as amino acids (and consequently peptides and proteins), polyamines, nucleic acids and vitamins. Nitrogen deficiency slows down yeast growth and the fermentation or may even result in a stuck fermentation, possibly due to the inhibition of the synthesis of proteins transporting sugar through the cell membrane to the interior of the

cells. It has been shown that an adequate supply of nitrogen increases yeast growth, provided the other essential yeast nutrients are not lacking (Malherbe *et al.*, 2007). Growth media are often supplemented with complex mixtures of amino acids. However, yeasts can also live on ammonium ions as sole nitrogen source. Ammonium sulphate is a commonly used nutrient in growth media because it provides a source of both nitrogen and sulphur. Ammonium assimilation by yeasts will depend mainly on the concentration of available ammonium ions and the intracellular amino acid pool (Walker, 2009).

4.1.1.4 Oxygen

Oxygen is consumed by the microorganisms for cellular growth and maintenance, biomass, CO_2, and water as products. During the fermentation there is a wide range of dissolved oxygen (DO) requirement among the yeast strains (Hornsey, 1999).

4.1.1.5 Phosphorus

Phosphorus requirements of yeast cells are met by the uptake of inorganic phosphate from the nutrient media. The phosphate taken up can be utilized for incorporation into major cell constituents such as phospholipids, nucleic acids and proteins, and is needed for the many transphosphorylation reactions in intermediary metabolism. The intracellular concentration of free phosphate is generally maintained at very low levels. The use of inorganic phosphate suggests that the utilization of phosphorus depends upon the physiological nature of the organism (Kumar and Sathyanarayana, 2001). Phosphate limitation has been shown to affect cell growth and biomass formation as well as directly affecting fermentation rate (Boulton *et al.*, 1996).

4.1.1.6 Sulphur

The uptake of sulphate ions is via an active process and most of it will occur early on during fermentation. Methionine is the most easily utilizable of the organic sulphur compound but even this is absorbed slowly during the initial hours of fermentation (Hornsey, 1999).

4.1.1.7 Inorganic ions

During fermentation, the concentration of Mg^{2+} and K^{+} is of most significance. Magnesium is fundamental to the metabolic and physiological function of the cell. Mg also plays a role in preventing cell death when the concentration of ethanol builds up within the cell, and has also been shown to improve the cells ability to withstand stress (Walker, 2000).

4.1.1.8 Potassium

Potassium contribute to the appropriate ionic environment for enzyme functioning. It makes a significant contribution to the internal osmotic potential (Jennings and Lysek, 1996). It is considered as the primary factor in maintaining the osmotic balance between the cell and the surroundings. The potassium content in the cells is dependent on the ionic strength of the growth medium. Potassium is taken up actively by yeast when there is a requirement of glucose or other sugars. The uptake of potassium by the cell is closely associated with the excretion of the H+ ions from the cell (Kurkdjian and Guern, 1989).

4.1.1.9 Trace Elements

4.1.1.9.1 *Calcium*

With regard to calcium there are conflicting views on the level of calcium ions required for yeast growth. Calcium is an important element

in yeast membrane structure and function. Its presence can stimulate yeast growth even though it is not strictly a growth requirement. Most of the influences of calcium are extracellular as far as the yeast is concerned and primarily involved in the maintenance of membrane and cell wall integrity. Calcium also plays an important role in the flocculation process (Hornsey, 1999)

4.1.1.9.2 *Zinc*

An essential nutrient, zinc, plays critical roles in a wide variety of biochemical processes. For example, zinc is a cofactor required for the function of over 300 different enzymes including representatives from all six major functional enzyme classes (Vallee and Auld, 1990). Zinc is also an important structural cofactor for many proteins including the ubiquitous zinc finger DNA binding proteins (Rhodes and Klug, 1993). Although zinc is essential, excess zinc can be toxic to cells. Therefore, while maintaining adequate levels of zinc for growth, cells must also control intracellular levels when exposed to excessive zinc concentrations (Gaither and Eide, 2001)

4.1.1.9.2 *Manganese*

The micronutrient, manganese, serves as an essential trace element and acts as a regulator of several intracellular enzymes. This has also been shown to be important in many other cell functions, most notably cell wall synthesis, sporulation and production of secondary metabolites (Shu and Johnson, 1948).

4.1.2 Culture medium for yeast

The development of an economically viable culture medium is necessary to produce yeast biomass on an industrial scale. The constituents

of a medium must satisfy the basic requirements for cell biomass and metabolite production, by providing an adequate supply of energy for biosynthesis and cell maintenance (Costa *et al.*, 2002). Designing the fermentation media is of critical importance because the media composition can significantly affect product concentration, yield, volumetric productivity, and ease the cost of downstream product separation (Baishan *et al.*, 2003).

Yeast acquires carbon and energy from their growth environment which include complex carbohydrates (starch), simple sugars (lactose, sucrose, and glucose) and sugar containing raw materials (molasses, corn syrup and cheese whey). In addition to carbon source, nitrogen source is also required in a medium. The nitrogen can be provided by addition of ammonium salts to the culture medium. The rich medium ingredients such as yeast extract, peptone, dextrose (YPD) are most commonly used for growing yeast under nonselective conditions. A good culture medium should contain all the essential nutrients needed for growth at optimal level. The choice of a good medium is virtually as important to the success of an industrial fermentation as is the selection of an organism to carry out the fermentation (Casida, 1968).

Many methodologies, like the single factor optimization technique, elemental composition analysis of biological materials, and statistically designed experiments, have been applied to formulate chemically defined media for various microorganisms (Spaargaren, 1996; Mantha *et al.*, 1998; Zang and Greasham, 1999; Shi *et al.*, 2006a; Preetha *et al.*, 2007). Some yeast species such as *Torulopsis* and *Candida* are also able to grow on methanol as sole energy and carbon source (Bekatorou *et al.*, 2006).

Minimal medium, also known as synthetic defined (SD) medium, supports the growth of yeast, which has no specific nutritional requirements. It contains yeast nitrogen base, ammonium sulphate, and dextrose (Bergman, 2001).

The composition of fermentation medium consisting of carbon sources, nitrogen sources, mineral salts, trace elements, peptides, amino acids, vitamins and/or other growth factors determines the chemical and nutritional environment of cells in a reactor and is thus vital for the effective manufacturing of products accumulated in the cells or secreted into the medium (Weuster-Botz, 2000).

In recent years increasing attention has been given to the conversion of food processing wastes in to valuable by – products such as yeast protein from pineapple cannery effluent (Nigam, 1998) prawn shell waste (Rishipal and Philip, 1998) and cane molasses (Singh and Satyanarayana, 2006). A number of agricultural and agro industrial waste products, including orange waste, mango waste, cotton stalks, kinnow-mandarin waste, barley straw, corn cops, rice straw, corn straw, onion juice, sugar cane bagasse (Nigam *et al*., 2000), cassava starch (Tipparat *et al*., 1995), wheat straw (Abou Hamed, 1993), banana waste (Saquido *et al*., 1981), capsicum powder (Zhao *et al*., 2010) and coconut water (Smith and Bull, 1976) have been used for the production of SCP and other metabolites. The raw materials used as substrate for industrial yeast biomass production are usually sorghum hydrolysate, sulfate waste liquor, prawn-shell wastes, dairy wastes, molasses stillage, starch, plant origin liquid waste, forestry and food waste by-products. *Candida utilis* proved its potential to utilize sulphite waste liquor from the paper pulping industry as the sole carbon source (Wiley *et al*., 1950).

Crude media are more likely to provide excess of toxic ions than to be deficient in required ions. Several of the better crude nutritive sources are in themselves complex mixtures of nutrients, supplying carbon and nitrogen compounds as well as microbial growth factors (Casida, 1968). Ornelas-Vale *et al*. (1977) succeeded in producing single cell protein from the nopal fruit using *C. utilis*. Single cell protein production from mandarin orange peel was studied using various yeast strains like *S. cerevisiae*, *C. utilis*, *Debaryomyces hansenii* and *Rhodotorula glutinis* by Nishio and Nagai (1981). *Kluyveromyces fragilis* strains were exploited for the production of yeast biomass from whey (Castillo, 1990). Manilal *et al*. (1991) were successful in aerobic treatment of cassava starch factory effluent with concomitant SCP production using a mixed culture of *C. utilis* and *Endomycopsis fibuliger*. Single cell protein production from *Schwanniomyces castellii* was optimized using cassava starch as carbon source (Hongpattarakere and Kittikun, 1995). Konlani (1996) optimized the cell yield of *Candida krusei* SO1and *Saccharomyces* sp. LK3G cultured in sorghum hydrolysate. Ram horn hydrolysate was used as carbon source for the single cell production of *C. utilis*, a significant waste product of the meat industry in Turkey (Kurbanoglu, 2001). Marine yeast *Cryptococcus aureus* G7a was suitable for single cell protein production from Jerusalem artichoke extract used as carbon source (Lingmei *et al*., 2007). Economic production of single cell protein from yeast *Debaryomyces hansenii*, *Kluyveromyces marxianus* and *Pichia stipitis* using non – detoxified breweries and spent grains hemicellulosic hydrolysate have been reported (Duarte *et al*., 2008).

Production of high quality edible protein from *Candida* grown on sugarcane molasses have been reported by Lawford *et al*. (1979). Many

workers have reported the utilization of molasses as carbon source in fermentation processes and in the production of lactic acid and citric acid (Zarowska *et al*., 2001; Young-Jung *et al*., 2004). Sugarcane molasses is the final run-off syrup from the sugar industry, which can be used after dilution as a cheap carbon source for fermentation process. It is rich in sugars, most in fermentable forms such as sucrose, fructose, raffinose and glucose. In molasses total residual sugars can amount to 50-60% (w/v). In addition to sucrose, there are both growth promoting components, inhibitors, heavy metals (Iron, zinc, copper, manganese, magnesium, calcium etc.) and alcohols (Gough *et al*., 1996). Molasses with other media ingredients such as different nitrogen sources (KNO_3, Urea, $(NH_4)_2SO_4$, $MgSO_4$, $CaCl_2$, KH_2PO_4, peptone and yeast extract were optimized in previous studies (Sarlin, 2005; Sajeevan, 2006) on the same strain of marine yeast *Candida* MCCF101. However, due to its wide scale application, the suitability of molasses for the growth of the yeast *Candida* MCCF101 was assessed in the present study. The recovery of such by-products can significantly reduce the costs of waste disposal, and SCP generation promises wide scale application in aquaculture, particularly as a feed supplement. The usage of such wastes as the sole carbon and nitrogen sources for the production of SCP by microorganisms could be simply attributed to their abundance and low cost.

4.1.3 Optimization

In developing a biotechnology-based industrial process, designing the fermentation media is of critical importance. The fermentation medium affects the product yield and volumetric productivity. It is also important to reduce the cost of the medium as much as possible, as this may affect the

overall process economics. Medium screening studies are very time consuming and expensive (Furuhashi and Takagi, 1984). For economy of effort and scale, different approaches have been used to rapidly identify the variables, which need to be controlled for optimizing production of useful product (Parra *et al.*, 2005).

The aim of optimization is to determine suitable reaction conditions for the respective biological system in order to maximize or minimize economically or technologically important process variables such as product concentration, yield, selectivity and raw material cost (Dirk, 2000).The optimization studies using response surface methodology (RSM), which is a mathematical and statistical technique is widely used for comparison and advanced optimization of medium constituents including carbon source, nitrogen source and inorganic compounds for single cell protein production (Lhomme and Roux, 1991; Jian and Nian-fa, 2007)

4.1.3.1 One factor at a time

One factor at a time is a close - ended system for fermentation process optimization. This method can be applied for optimization of medium components as well as for process condition and it is based on the classical method of one independent variable while fixing all others at a certain level (Alexeeva *et al.*, 2002; Paditar *et al.*, 2005; Ahamed *et al.*, 2006). This strategy has the advantage that it is simple, easy and the individual effects of the medium components and process condition can be seen on graphs (Kar *et al.*, 1999; Kumar *et al.*, 2003). But the limitations of this method are its incapability of reaching the true optimum especially because of interactions among the factors, extremely time consuming and expensive for large number of experiments. These limitations of single

factor optimization process can be eliminated by optimizing all the affecting parameters collectively by statistical experimental design. However, one factor at a time method has been the most popular method for improving the fermentation medium and process condition (Wang *et al*., 2008).

4.1.3.2 Factorial Design

In this method level of parameters are independently varied, each factor at two or more levels. The effects that can be attributed to the factors and their interactions are assessed with maximum efficiency in factorial design. Moreover, it allows for the estimation of the effects of each factor and interaction. The optimization procedures are facilitated by construction of an equation that describes the experimental results as a function of the factorial level. A polynomial equation can be constructed in the case of a factorial design where the coefficient in the equation is related to the effects and interactions of the factors (Panda *et al.*, 2007).

4.1.3.3 Plackett-Burman design

Plackett–Burman design is a well-established, widely used statistical design technique for the screening of key factors from a large number of medium components by a small number of experiments (Escamilla *et al.*, 2000; Xu *et al.*, 2003). This design is useful to screen out important factors which influence the fermentation process (Naveena *et al.*, 2005). This technique allows for evaluation of n variables by n+1experiments where n+1 must be a multiple of 4 e.g., 8, 12, 16, 24 etc. Each variable is represented at two levels, high and low, denoted by (+) and (-), respectively. The number of positive and negative signs per experiment or trial are (n+1)/2 and (n-1)/2,

respectively. Each column should contain equal number of positive and negative signs (Singh and Satyanarayana, 2006).

The Plackett- Burman design was based on the following first order model:

$$Y = \beta_0 + \sum \beta_i x_i \quad \text{----------(1)}$$

Where, Y represents the yield (biomass), β_0 is the model intercept, β_i is the linear coefficient and x_i is the level of the independent variable (Liu *et al.*, 2010; Mukherjee and Rai, 2011).

Any factor not assigned to a variable can be designated as a dummy variable. The incorporation of dummy variable in to an experimental design makes it possible to estimate the variance of effects (Plackett and Burman, 1946). The main disadvantage of these designs is that they consider only first order effects and ignore interactions. It is useful in the first step of screening of significant medium components affecting biomass production and is useful in decreasing the number of variables and number of experiments in further optimization step. Thus, optimum performance has been determined using response surface methodology.

4.1.3.4 Response Surface Methodology

The application of statistical experimental design techniques in fermentation process development can result in improved product yields, reduced process variability, closer confirmation of the output response to nominal and target requirements and reduced development time and overall costs (Elibol, 2004). Conventional practice of single factor optimization by maintaining other factors involved at an unspecified constant level does not

depict the combined effect of all the factors involved. This method is also time consuming and requires a number of experiments to determine optimum levels, which are unreliable. RSM can eliminate all these limitations (Elibol, 2004).

RSM seeks to identify and optimize significant factors with the purpose of determining what levels of factors maximize the response (Singh and Satyanarayana, 2006). It is possible to separate an optimization study using RSM into three stages. The first stage is the preliminary work in which the determination of the independent parameters and their levels are carried out. The second stage is the selection of the experimental design and the prediction and verification of the model equation. The last one is obtaining the response surface plot and contour plot of the response as a function of the independent parameters and determination of the optimum points. The contours of a response surface optimization plot show lines of identical response. Response means, the results of an experiment carried out at particular values of the variables, and the area within the axes is the response surface.

Preliminary screening experiments are useful to identify the independent parameters. Factorial designs may be used for this purpose. After the identification of important parameters, the direction of improvement is determined and the levels of the parameters are identified. Determination of these levels is important because the success of optimization process directly relates to these levels. After screening experiment, selection of the experimental design should be taken into account in choosing a response surface design. These designs differ from one another with respect to their selection of experimental points, number of runs

and blocks. After selection of the design, the model equation is defined and coefficients of the model equation are predicted. When the regression coefficients are obtained, the estimated response can be easily calculated using model equation. Usually the behavior of the system is unknown, so one must check whether the model fits well to the experimental data. The overall predictive capability of the model is commonly explained by the coefficient of determination (R^2) (Bas and Boyaci, 2007). To construct a contour plot the results (response) of a series of experiments employing different combination of variable are inserted on the surface of the plot at the points delineated by the experimental conditions giving the same results, joined together to make a contour line (Kumar *et al.*, 2004).

Gonen and Aksu (2008) used RSM in the evaluation of growth and copper (II) bioaccumulation properties of *Candida utilis* in molasses medium. He and Tan (2006) used RSM to optimize culture medium for production of lipase with *Candida* sp.99-125. Soni *et al.* (2007) optimized the medium components for carbonyl reductase production by a novel yeast strain *Candida viswanathii* using response surface methodology.

Optimization for biomass production requires that the specific growth rate and biomass yield in the fed-batch process be as high as possible (Van Hoek *et al.,* 1998). Rajoka *et al.* (2006) developed and optimized a fermentation process for the production of SCP by culturing *Candida utilis* on rice polishing in 14 and 50L aerated fermentors. Cheese whey fermentation for the production of SCP using the yeast *Kluyveromyces fragilis* was developed by Ghaly *et al.* (2005).

4.1.4 Fermentation Technology

This complex microbial process probably represents the oldest form of biotechnological application of a microorganism and has been used by humans for several thousand years (Samuel, 1996). Fermentation processes are popularly operated to regulate substrate or product concentration in the reactor. The design of optimal control strategies for fermentation process is a growing field in biotechnology. The economics of fermentation are greatly affected by the amount of substrate and oxygen needed to produce the required amount of biomass. The most common requirement in the study of a fermentation process is the development of a suitable medium for obtaining the maximum yield of the desired product with complete utilization of the carbon source to reduce pollution encountered in the spent broth (Sreekumar *et al.*, 1999). In fermentation process optimization of different combinations and sequence of process conditions and medium components are needed to be investigated to determine the growth condition that produce the biomass with the physiological state best constituted for product formation (Panda *et al.*, 2007). Difficulties in the study of yeasts under real industrial conditions can be overcome by the use of bench-top trials reproducing both industrial biomass yield and growth rate, which allow the application of molecular tools for the understanding and improvement of yeast behaviour (Perez-Torrado *et al.*, 2009). The biomass and secondary metabolites production was affected by many typical fermentation factors such as composition of the medium, carbon: nitrogen ratio, pH of the culture medium, temperature, type of aeration, inducers and presence of some precursors (Bhima *et al.*, 2012).The literature review has considered the use of fermentation process for biomass production and other metabolite products from various yeasts

using various media (Nishio and Nagai 1981; Valero *et al.*, 1991; Meyer *et al.*, 1992; Hongpattarakere and Kittikun 1995; Bell *et al.*, 2001; Ueno *et al.*, 2002; Berthels *et al.*, 2004; Mendes-Ferreira *et al.*, 2004; Gardner *et al.*, 2005; Kuyper *et al.*, 2005; Oliveira *et al.*, 2006; Bohlscheid *et al.*, 2007)

Marine yeast would be a better candidate for fish meal on large scale production according to their easy cultivation in the fermentor and high cell density.

In this chapter optimization of the medium to be used for the growth of *Candida* MCCF 101 by studying the influence of selected nutrients such as carbon (as primary energy source), nitrogen (for protein synthesis), phosphorus (needed for ATP production), magnesium (an important enzymatic cofactor), calcium (maintenance of membrane and cell wall integrity) and yeast extract (stimulate cell growth) for the single cell protein production. Response surface methodology was used to obtain a predicted model and this model was used for optimizing the fermentation medium, formulation of fermentation process conditions and for better understanding the fermentation process. Besides, fermentation employing a commercial substrate, molasses, has also been accomplished.

4.2 Materials and Methods

4.2.1 Selection of medium for optimization

Medium reported by Tobajas *et al.* (2003) which was considered to be the best for biomass production of *Candida* was selected in this experiment for optimization. Mineral based medium had the following composition (gL^{-1}): D-glucose - 10; $(NH_4)_2SO_4$ -5; KH_2PO_4 -5; $MgSO_4.7H_2O$ - 0.5; $CaCl_2$ - 0.13; yeast extract - 0.5.

4.2.2 Preparation of inoculum

Mineral based medium was solidified with 2% agar and made into slants. The yeast isolate *Candida* MCCF 101 was streaked on to slants and incubated at 26 ± 1°C for 48 hours. The cells were harvested at the logarithmic phase using 0.5% sterile saline. Optical density of the culture suspension was taken at 540nm in a UV-Vis spectrophotometer (Shimadzu UV-1601) and absorbance was adjusted to 0.1 by appropriate dilution and 100µl of this suspension was used as the inoculum. At this absorbance, the inoculum contained approximately 5.57×10^5 cfu mL^{-1}.

4.2.3 Shake flask experiment

4.2.3.1 Optimization of mineral based medium

4.2.3.1.1 *Optimization of carbon source*

Carbon source was varied in the first set of experiments. Different carbon sources used were glucose (1%), sucrose (1%), starch (1%), xylose (1%), lactose (1%), and maltose (1%). The experiments were carried out in 250 mL Erlenmeyer flasks with a working volume of 50 mL. The flasks were inoculated with 0.1mL cell suspension and incubated at 26.3°C for 72 hours on a rotary shaker at 100rpm and growth was measured from the absorbance at Abs_{540nm} in a spectrophotometer (UV-1601, Shimadzu Corporation, Tokyo, Japan).

4.2.3.1.2 *Optimization of nitrogen source*

The nitrogen source was altered in this set of experiments. Nitrogen sources selected were $(NH_4)_2SO_4$ (1%), KNO_3 (1%), NH_4Cl (1%), $(NH_4)_2HPO_4$ (1%) and NH_4NO_3 (1%). The experiments were carried out in 250mL Erlenmeyer flasks containing 50 mL medium. Flasks were inoculated as

above and incubated at 26.3°C for 72 hours. Growth was measured at Abs_{540}nm.

4.2.3.2 Optimization of molasses concentration

Varying concentrations of molasses were used to prepare the molasses medium (0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5% w/v). After inoculation with 0.1mL cell suspension (5.57×10^5 cfu mL^{-1}) the flasks were incubated at 28±1°C on a rotary shaker at 100rpm and growth was measured by absorbance at Abs_{540nm} in a spectrophotometer (UV-1601, Shimadzu Corporation, Tokyo, Japan).

4.2.4 Experimental Design

Optimization of the medium was carried out in two steps. The first step was to determine the effect of each constituent on biomass production of *Candida* MCCF 101 while the second step was to further adjust the levels of media constituents and study the effect of interaction between the constituents to attain maximum biomass.

4.2.4.1 First step optimization

Initial screening of factors was carried out by one-variable-at-a-time approach in shake flasks in order to find out ranges of the medium components that have to be used for further optimization. Mineral based media with different concentrations of maltose (1.0-5.0%), NH_4Cl (0.02-5.0%), KH_2PO_4 (0.05-0.60%), $MgSO_4.7H_2O$ (0.00-0.07%), $CaCl_2.6H_2O$ (0.00-0.06%) and yeast extract (0-0.07%) were prepared and inoculated with *Candida* MCCF 101 to find out the optimal range. The concentration of one ingredient was varied over the given range, while, all the other ingredients were kept constant at their standard levels. The optimum ranges of

each of the media components were used for analysis using the Plackett-Burman design.

4.2.4.2 Screening of significant variables using Plackett and Burman design

Plackett and Burman design is useful in the first step of screening of significant media components affecting biomass production and is useful in decreasing the number of variables and the number of experiments in further optimization step. The range of maltose, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.6H_2O$ and yeast extract were fixed on the basis of the first step optimization.

The Plackett and Burman design allows the evaluation of N (11) variables in N+1(12) experiments with each variable represented at two levels, high (+1) and low (-1). The design consisting of a set of 12 experiments was used to determine the relative importance of 11 factors that influenced the biomass production by *Candida* MCCF 101.The variables chosen for the present study were maltose, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2.6H_2O$ and yeast extract (Table 1). Total of six components were selected for the study with each variable being represented at two levels, high and low denoted by (+) and (-), respectively and five dummy variables in 12 trials, each row representing a trial and each column representing an independent or dummy variable (Table 2). The design was developed using Design Expert version (6.0.9), Stat-Ease Inc, Minneapolis, MN, USA. All the trials were carried out in triplicates. The average biomass production of each trial was used as the response variable. Regression analysis determined the variables that had significant ($p<0.05$) effect on biomass production and these variables were evaluated in further optimization experiments.

4.2.4.3 Optimization of media components by Face Centered Central Composite Design (FCCCD) of the RSM

The process parameters such as maltose, $MgSO_4.7H_2O$ and yeast extract were optimized statistically using the full factorial Face Centered Central Composite Design (FCCCD) of the RSM and it was applied to find out the optimum levels of significant variables and the effects of their interaction on biomass production. A 2^3 FCCCD of 3 factors with an alpha value of 1, including six centre points with a set of 20 experiments were carried out. Each independent variable such as maltose, $MgSO_4.7H_2O$ and yeast extract was studied at three different levels (low, medium and high,) coded as -1, 0 and +1 (Table 3). A total of 20 trials were employed with six replicates at the centre point. The central coded value was defined as zero for all variables. Each run was performed in triplicate and the average biomass production was taken as the experimental value of the dependent variable or response (Y), while predicted values of the response were obtained from quadratic model fitting. A multiple regression analysis of the data was carried out to define the response in terms of independent variables. The experimental results of RSM were fitted with the response surface regression procedure using the following second order polynomial equation:

$$Y= b_0 + b_1A+b_2B+b_3C+b_{12}AB+b_{23}BC+b_{13}AC+b_{11}A^2+b_{22}B^2+ b_{33}C^2 \text{--------} \quad (2)$$

Where 'Y' is the estimated response, 'b_0' is a constant, 'b_1, b_2, b_3, b_{11}, b_{22}, b_{33}, b_{12}, b_{23}, b_{13}' are the coefficients for each term and 'A, B and C' are the independent variables.

The statistical significance of the model equation and the model terms were evaluated via. Fisher's F test. The quality of the fit of the second order

polynomial model equation was expressed the coefficient of determination, R^2 and the adjusted R^2. The fitted polynomial equation was then expressed as three dimensional surface plots to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

The response surface graphs were obtained to understand the effects of the variables individually and in combination and to determine their optimum levels for maximum biomass production. The data on biomass production was subjected to analysis of variance (ANOVA). The software, Design Expert (version 6.0.9, Stat-Ease Inc, Minneapolis, MN, USA) was used for the experimental design, data analysis and quadratic model building.

4.2.4.4 Validation of the model

The validation of the statistical model was done by carrying out the experiment at optimum values of the mineral based media parameters as determined from the model. Samples were withdrawn at desired intervals and biomass estimated as described earlier. The experiments were carried out in triplicates and results were expressed as mean ± standard deviation. The experimental values were subsequently compared with the predicted values by the model.

4.2.5 Scale up of production:

4.2.5.1 Biomass production in 5L laboratory scale fermentor

4.2.5.1.1 *Laboratory scale fermentor containing mineral based medium:*

The fermentation was carried out in a 5L fermentor (Biostat- B-lite bench top fermentor; Sartorious, Germany) containing 3L culture medium

(Fig.1). Fermentation conditions obtained as optimum through RSM were maltose-50.8gL^{-1}, $MgSO_4.7H_2O$-1.8gL^{-1} and yeast extract -18.0gL^{-1}, pH 6.51, temperature 26.3^oC and salt content 0ppt with minimum concentration of insignificant factors such as KNO_3 - 2.5gL^{-1}, KH_2PO_4 - 2gL^{-1} and $CaCl_2.6H_2O$ - 0.1gL^{-1}. In addition, aeration 2.5LPM and agitation of 300rpm (revolutions per minute) were also provided. Silicon antifoaming reagent (Himedia Laboratories limited, Mumbai) at 1% concentration was prepared and autoclaved to use for foam control. The vessel was sterilized at 121^oC for 60min. Inoculated with 0.1 % (v/v) yeast suspension in to the modified mineral based medium and maintained for 72 hours. Cell biomass was harvested at exponential phase and biomass quantified.

Air was provided from oil free pump (Model OF01080, Elgi Equipments Limited, Coimbatore) with a working pressure of 8 kgf/cm^3. Rotameter controlled the air flow rate in to the fermenter vessel. A sterile filter (0.22μm) was used as a bridge between the tubing from the rotameter and that connected to the air sparger of the fermentor. A second filter on the exit gas cooler stopped the microbes from releasing into the laboratory air as the gas left the fermentor under a slight positive pressure.

4.2.5.1.2 *Laboratory scale fermentor containing molasses based medium:*

The fermentation was carried out in a 5L fermentor containing 3L medium containing 3.5% molasses (Fig.2) under optimum conditions such as pH 6.51, temperature 26.3^oC and salt content 0 % following response surface methodology. In addition, aeration at the rate 2.5LPM and agitation at 300rpm were also provided. Biomass production was monitored at regular intervals of 24 h for 72 h.

4.2.5.2 Biomass production in 100L Fermentor:

100-L insitu sterilizable fermentor (Scigenics PVT Ltd Chennai India) containing 75L culture medium (Fig.3) was run maintaining the same conditions determined from the Response Surface Methodology. Cell biomass was harvested at the exponential phase, biomass quantified and cell suspension was autoclaved at 10 Lbs for 10 min in fermentor for inactivating yeast.

4.2.6 Calculation of production cost

Calculation of production cost of single cell protein *Candida* MCCF 101 in 100L pilot scale fermentor using mineral based medium was made. The procedure involved estimation of cost of substrates, other operating expenses, capacity of the plant, labour charge and cost of downstream process for the yield obtained.

4.3 Results

4.3.1 Shake flask experiment

The selection of optimum range of each medium component for obtaining maximum biomass of *Candida* MCCF 101 was carried out in this set of experiments.

4.3.1.1 Optimization of mineral based medium ingredients

A number of different carbon and nitrogen sources were tested and biomass production monitored during the experiment. Among the different substrates tested as carbon source, maltose produced the highest biomass under the same operating conditions. In the case of nitrogen source, the shake flask experiment showed NH_4Cl as the most favoured nitrogen source for the same. The results are summarized in Fig 4 and Fig 5.

4.3.1.2 Optimization of molasses based medium ingredients

Result obtained in the present study suggested that the optimum concentration of the molasses was 3.5 % for better growth/production of *Candida* MCCF 101 (Fig.6). Increasing concentration of molasses from 0.1 to 3.5% proportionately increased the production of yeast 6.8 ± 0.12gL^{-1} dry weight whereas higher concentrations did not produce significant increase in biomass production.

4.3.2 One-dimensional screening

One dimensional screening results were used for the regression analysis of the quantities of maltose, NH_4Cl, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$ and yeast extract. Following are the significant ($p<0.05$) ranges selected of the various media components, such as maltose (2-5%), NH_4Cl (0.025-0.3%), KH_2PO_4 (0.2 - 0.6%), $MgSO_4.7H_2O$ (0.030-0.070%), $CaCl_2.6H_2O$ (0.0125-0.025%) and yeast extract (0.01 - 0.06%) (Fig.7 a-f), and were used for the application of RSM.

4.3.3 Screening of media components using Plackett and Burman Design

Plackett–Burman Design was used for screening media components employing limited number of experiments. Total of six components were selected for the study with each variable being represented at two levels, high and low denoted by (+) and (-), respectively and five dummy variables in 12 trials, each row representing a trial and each column representing an independent or dummy variable The 11 selected variables along with their corresponding experimental and predicted values are shown in Table 4. The effect of each variable along with the p value and F level is presented in Table 5. The adequacy of the model was determined. The "Model F

value" of 25.39 and low probability value of 0.0112 [(Prob>F)] implied that the model was significant. The p values were less than 0.05 indicating that the model terms were significant. The Prob>F < 0.05 was considered to have significant effect on biomass production. Thus maltose (p = 0.0049), lactose (p = 0.0290), $MgSO_4.7H_2O$ (p = 0.0083) and yeast extract (p = 0.0043) were significant factors. The magnitude of coefficient of each variable indicated the intensity of its effect on the studied response. Thus, yeast extract (29.7%) was found to be the most influential factor on biomass production followed by maltose (27.1%) and $MgSO_4.7H_2O$ (18.3%). They were chosen for the next round of optimization using FCCCD. Contribution of lactose (7.5%) was found to have less influence, and therefore eliminated from the medium (Table 6).

4.3.4 Optimization of media components by Face Centered Central Composite Design (FCCCD) of RSM

The Face Centered Central Composite Design (FCCCD) was applied in the optimum vicinity to locate the true optimum concentrations of maltose, $MgSO_4.7H_2O$ and yeast extract for biomass production of *Candida* MCCF 101. The levels of the variables for the FCCCD experiments were selected according to the results of the previous experiments using Plackett–Burman Design. The FCCCD design matrix and the corresponding experimental data are shown in Table 7. The FCCCD matrix was statistically analyzed by the analysis of variance (ANOVA) and the results shown in Table 8. The statistical significance of the second-order model equation was evaluated by the F-test analysis of variance which revealed that this regression is statistically significant (p < 0.0004) at 99% confidence level. The values of F- statistics was found to be 136.54. High model F value with a very low probability values [(Prob>F)] less than

0.0001 implied that the model was highly significant. The 'lack of fit' was insignificant [(Prob>F)] = 0.2197. The fit of the model was checked by the coefficient of determination R^2, which was determined to be 0.9919, indicating that 99% of the variability in the response could be explained by the model. "Adj R^2" of 0.9847 and "Pred R^2" of 0.9548 reasonably agreed indicating a good agreement between the experimental and the predicted values for biomass production. Adequate precision measures signal to noise ratio and a ratio greater than four were desirable (Wang and Lu, 2004). Adequate precision obtained in this experiment was 13.323 which suggested that the model could be used to navigate the design space.

The significance of the regression coefficients of the model indicated that the linear terms A, B and C, the quadratic terms A^2, B^2 and C^2 and interaction terms AC and BC were significant model values (Prob>F< 0.05) on biomass production. In this model maltose (A), $MgSO_4.7H_2O$ (B) and yeast extract (C) had significant effect on biomass production by *Candida* MCCF 101. The interaction effects between maltose and yeast extract (AB) and $MgSO_4$ and yeast extract (BC) were also found to be significant.

The experimental results of the FCCCD design were fitted with the second-order polynomial equation

$$\text{Biomass (Y)} = 0.019761 + 0.51869A + 22.18707B + 4.13816C + 0.45089AB + 4.02838BC + 0.10122AC - 0.055341A^2 - 99.07236B^2 - 1.21777C^2 \quad (2)$$

where A, B and C were the concentrations of maltose, $MgSO_4.7H_2O$ and yeast extract respectively.

The response surface and contour plot showed the interaction effect of factors such as maltose, $MgSO_4.7H_2O$ and yeast extract on biomass production. The response surface plot was used to determine the optimum concentration of each factor and the interaction effect of maltose and yeast extract (AC) and $MgSO_4.7H_2O$ and yeast extract (BC) and maltose and $MgSO_4.7H_2O$ (AB) on biomass production. The contour plot was elliptical indicating that the interaction between maltose and yeast extract (AC) and $MgSO_4.7H_2O$ and yeast extract (BC) were significant. The interaction between maltose and $MgSO_4.7H_2O$ was not significant. The response surface and contour plots and the interaction effect of each factor at optimum biomass production are shown in Fig.8 (a-c). The points given for maximum biomass productions were maltose 50.8gL^1, $MgSO_4.7H_2O$ 1.8gL^{-1} and yeast extract 18gL^{-1}. In this set-up, the model predicted a maximum biomass concentration of 8.15gL^{-1}.

4.3.5 Validation of optimum condition

The model was validated by repeating the experiments in triplicate under optimum conditions in shake flasks. The solution obtained from the model is given in Table 9. The experimental value of biomass production (7.9± 0.07 gL^{-1}) was closer to the predicted value of 8.15gL^{-1} validating the model. By using FCCCD for optimization of the media ingredients, the yield could be increased from 3.89 ± 0.09gL^{-1} to 7.9± 0.07 gL^{-1} compared to the biomass production under the laboratory conditions prior to optimization by RSM.

4.3.6 Fermentation Experiments

4.3.6.1 Biomass production in mineral based medium

The increased biomass yield of *Candida* MCCF 101 obtained in the present study is attributed to the optimization of significant components in

the mineral based medium. Further scale up studies were done in 5L and 100L fermenters. Using the experimental set up of laboratory and pilot scale production the optimized media components were used to scale up the production, standardization of downstream process and development of bench scale product of *Candida* MCCF 101. The Optimization of the media components were accomplished by Face Centered Central Composite Design (FCCCD) of the RSM. The optimized experimental conditions using RSM were adopted viz., maltose 50.8gL^{-1}, $MgSO_4.7H_2O$ 1.8$gL^{-1}$and yeast extract 18gL^{-1} and the samples were withdrawn once in every 24 hrs to estimate the growth of the yeast. When maximum biomass was attained, yeast was harvested (at 72 hrs). The biomass attained was divided into two lots. One lot was transferred to small bottles and stored at room temperature for use as live yeast in subsequent experiments. The other lot was retained in the fermentor and steamed at 10 Lbs for 10 min for inactivating the yeast. This was harvested into small bottles and stored at room temperature to be used as inactive yeast in subsequent experiments. The maximum biomass production attained was 16.06gL^{-1} (Fig.9) in 5L lab scale fermentor (Fig.10) and 18gL^{-1} (Fig.11) in pilot scale fermentor in dry weight.

4.3.6.2 Biomass production in molasses based medium

Biomass production of *Candida* MCCF 101 was determined in 5L laboratory scale fermentor containing molasses (3.5%) based medium (Fig. 12). It attained growth of 0.55± 0.11gL^{-1} at 24 hrs and reached its maximum with in 72 hrs (6.8 ± 0.12gL^{-1} dry weight) (Fig. 13).

4.3.1.7 Cost of SCP production

Production cost of *Candida* MCCF 101 in pilot scale fermentor using mineral based medium was calculated and summarized in Table 10. The

total cost for the production of yeast suspension in pilot scale fermentor was Re 1.0 mL^{-1}.

4.7 Discussion

In biotechnology-based industrial processes, the formulation of cultivation media is of critical importance as their composition affects product concentration, yield and volumetric productivity. It is also important to reduce the costs of the medium as this may affect the overall process economics (Souza *et al.*, 2006). Yeast requires carbon and nitrogen sources and also a range of metals such as magnesium, sodium, potassium, iron, zinc, copper and manganese and other inorganic nutrients such as chloride, sulphur and phosphate. Literature available revealed various media such as synthetic, complex and crude used for general cultivation of yeasts (Meyer *et al.*, 1992; Hongpattarakere and Kittikun, 1995; Casas *et al.*, 1997; Vazquez and Martin, 1998; Zhang *et al.*, 2004; Rosma and Cheong, 2007; Duarte *et al.*, 2008).

Purpose of this investigation was to study the effects of media ingredients on biomass production of the marine yeast *Candida* MCCF 101 in mineral based medium. The mass production of *Candida* MCCF101 as SCP seems to be an interesting solution to the growing problem of protein-rich supplement for aquaculture feeds. To be economically feasible, it is necessary to engineer optimum culture conditions for maximum biomass productivity. In microbial fermentations, the cost of the fermentation medium can account for almost 30% of the total cost (Miller and Churchill, 1986).

The present study using shake flasks experiment enabled to find the suitable carbon and nitrogen sources for the maximal production of

Candida MCCF 101. For determining the effects of carbon and nitrogen sources on the biomass production, cultivations were performed in mineral based medium with 1 gL^{-1} of different carbon source and 1 gL^{-1} of nitrogen sources. Among the various sources studied, the highest biomass yield of *Candida* MCCF 101 was obtained on incorporating maltose as single carbon source and NH_4Cl as the nitrogen source. Glucose, sucrose, starch, xylose and lactose were found to be poor carbon sources for *Candida* MCCF 101. Accordingly, maltose and NH_4Cl were chosen as the carbon and nitrogen sources for further experiments.

One dimensional screening (initial screening experiment) of different media components was carried out in order to find the significant ranges of medium components affecting the biomass production. From this method the individual effects of medium components could be seen on a graph without the need to revert to more sophisticated statistical analyses. The experimental data revealed that the *Candida* MCCF 101 had significant biomass production at wide ranges of maltose (2-5%), NH_4Cl (0.025-0.3%), KH_2PO_4 (0.2-0.6%), $MgSO_4.7H_2O$ (0.030-0.070%), $CaCl_2.6H_2O$ (0.0125-0.025%) and yeast extract (0.01%-0.06%). The selected ranges of these factors were further screened by Plackett-Burman design in order to find the significant factors affecting the biomass production.

Plackett - Burman design tests only two levels of each medium component, and it cannot determine the exact quantity of each constituent required for the medium. However, it indicates the importance of each constituent and how it tends to affect the growth and product formation. But this method frequently fails to locate the region of optimum response because the joint effects of factors on the response were not taken into

account in such procedures. When more than five independent variables are to be screened, the Plackett-Burman design is used to find out the most significant variables influencing the response (Stanbury *et al.*, 1997). Whether a variable has an influence or not, effects are presented in a normal one factor plot. It can be seen that the significant concentration of maltose (p = 0.0049), lactose (p = 0.0290), $MgSO_4.7H_2O$ (*p* = 0.0083) and yeast extract (p = 0.0043) have positive effects on biomass production of *Candida* MCCF 101. But NH_4Cl, KH_2PO_4 and $CaCl_2.6H_2O$ have negative effects on the biomass production. Based on the overall percentage contributions of the factors, the impact of NH_4Cl, KH_2PO_4 and $CaCl_2.6H_2O$ were found to be negligible and also the confidence level of these factors were found to be low, and therefore these components were removed from the media during further experiments. With the help of relative ranking, maltose, $MgSO_4.7H_2O$ and yeast extract within the tested limits were selected for further optimization, which had significant effects on biomass production.

Statistical designs employing minimum number of experiments to determine optimal concentrations of media components have an advantage. The RSM applied for the optimization of biomass production of *Candida* MCCF 101in this investigation suggested the importance of a variety of factors at different levels. The FCCCD of RSM supports recognizing the effects and interaction on the production of yeast. The analysis of variance (ANOVA) of the quadratic regression model demonstrated that the model was highly significant, as evidenced from the Fisher F test (F – 136.54) with a very low probability value (P > F < 0.0001). The correlation measures for the estimation of the regression equation were the multiple

correlation coefficients (R) and the determination coefficient (R^2). Coefficient of determination (R^2) was defined as the ratio of the explained variation to the total variation and was a measure of fit (Haber and Runyon, 1977). It gave a measure of how much the variability in the observed response value could be explained by the experimental factors and their interactions on biomass production of yeast. The closer the value of R is to 1, better is the correlation between the observed and the predicted values. Here, the value of the determination coefficient (R^2- 0.9919), being a measure of the "goodness of fit" of the model, it indicated that sample variation of 99.19 % for biomass was attributed to the independent variables and only 0.008 % of the total variation was not explained by the model. When the R^2 is large, then, the regression has accounted for a large proportion of the total variability in the observed value of Y which favored the regression model equation (Rai and Mukherjee, 2010).The adjusted R^2 value (0.9847) was also very high, making the model very significant. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. A relatively low value of the coefficient of variation (CV) indicated improved precision and reliability of the experiment. Here a lower value of CV (5.5%) indicates a greater reliability of the experiments performed. An 'adequate precision value' is used to measure the ratio of signal (controllable) to noise (uncontrollable), and a value of greater than 4 is generally desirable for a model to be a good fit (Wang and Lu, 2004; Oskouie *et al.*, 2008). Here, the value of ratio, 30.639 suggests that the polynomial quadratic model is of adequate signal, and can be used to navigate the design space (Khuri and Cornell, 1987). If the model has a significant lack of fit, then it is not a good predictor of the

response and should not be used. In this model, the 'lack of fit' ($p = 0.2197$) was found to be not significant.

The significant factors having influence on biomass production of *Candida* MCCF 101 in the mineral based medium were maltose (A), $MgSO_4.7H_2O$ (B) and yeast extract (C).The linear terms of A, B, C are significant terms. The quadratic terms of A^2 (maltose), B^2 ($MgSO_4.7H_2O$) and C^2 (yeast extract) were also found to be significant. If the factor is significant at the quadratic level, it can act as a limiting factor (Elibol, 2004). Interaction between maltose and yeast extract (AC) as well as $MgSO_4.7H_2O$ and yeast extract have significant effects ($p < 0.05$) on the biomass production of marine isolate *Candida* MCCF 101. In this case linear, quadratic and interaction effects of yeast extract were significant in playing an important role in biomass production. For the mass production of *Rhizobium*, yeast extract solution (200mL L^{-1}) and cheap carbon source, molasses, (15gL^{-1}) were found suitable (Nandi and Sinha, 1970). Nancib ***et al.*** (2005) reported an increase in lactic acid production from date juice by the use of yeast extract in the medium.

In the present study the experimental results clearly showed that the biomass production of *Candida* MCCF 101 was dependent mainly on maltose, $MgSO_4.7H_2O$ and yeast extract concentrations. It is also evident from the experimental results that maltose and $MgSO_4.7H_2O$ were probably enhancing the growth of the marine yeast isolate *Candida* MCCF 101. Yeast extract had significant positive effect on biomass production. The concentration of carbon source is crucial to a fermentation process. Media ingredients affect both production rate and yield, and cost of the whole process (Akerberg and Zacchi, 2000). High concentration of carbon source

can promote the production rate and improve process efficiency as biomass of yeast are mainly contributed by carbon, hydrogen, oxygen and nitrogen sources from the provided medium (Bamforth, 2005). Postec *et al.* (2005) observed that total elimination of yeast extract led to a dramatic decrease in cell concentration demonstrating that growth on maltose alone is not possible. Hence, when the biomass yield increased with nitrogen supplementation, substrate usage also increased concurrently. In this study, maximum biomass production was obtained at maltose concentration of $50.8gL^{-1}$. Magnesium plays a key role in metabolic control, growth and cell proliferation, glycolytic pathway and subsequently ethanol production. The concentration of essential cations like Mg+ is suboptimal for yeast growth and metabolism in molasses (Walker *et al.*, 1994). Magnesium directly affects sugar catabolism and consequently the fermentative activity (Jones *et al.*, 1981). The result of response surface methodology showed that maltose and $MgSO_4.7H_2O$ are the significant interaction factors and $MgSO_4.7H_2O$ was kept at its optimal value of $1.8gL^{-1}$.Yeast extract is the most commonly used nitrogen source in fermentation as it provides convenient growth factors for microbial growth (Nancib *et al.*, 2001). Nitrogenous compounds are used by yeast to produce structural and functional proteins that result in increased yeast biomass and the production of enzymes that facilitate many biochemical changes occurring during yeast fermentation (Spayd and Andersen-Bagge, 1996). The results obtained in this study showed that organic nitrogen such as yeast extract supported rapid growth and high cell yield of the *Candida* MCCF 101.Yeast extract is good for many microorganisms because it contains amino acids and peptides, water soluble vitamins and carbohydrates (Crueger and Crueger, 1993; Jackson *et al.*, 1998; Costa *et al.*, 2002). Yeast extract probably

contains nutrients that satisfy minimal requirement for growth of *Candida* MCCF 101.The result of RSM obtained in mineral based medium contains 18 gL^{-1} of yeast extract.

The contour plot was a good tool to separate the effect of interactions and to find the optimal conditions between two factors. Response surface and contour plots provide a method to visualize the relation between the response and experimental levels of each variable, and the type of interactions between the test variables (Sen and Swaminathan, 1997). In this investigation it provided a better understanding of the interactions between the mineral based medium substrates and their effects on biomass production of *Candida* MCCF 101. Graphs are given here to highlight the roles played by various factors in the final yield of the yeast. The shapes of contour plots indicate the nature and extent of the interactions. Shape of the contour plot, elliptical or circular indicates whether the mutual interactions between the corresponding variables are significant or not. Prominent interactions are shown by the elliptical nature of the contour plots while less prominent or negligible interactions would otherwise be shown by the circular nature of the contour plots. (Moyoa *et al.*, 2003; Dutta *et al.*, 2004; Wang and Lu, 2004). The 2D contour plot results indicated the interaction effect between maltose and yeast extract (AC) and $MgSO_4.7H_2O$ and yeast extract (BC) and found to be most significant for biomass production.

Verification of the predicted values of biomass was conducted using optimal for medium ingredients of maltose (50.8gL^{-1}), $MgSO_4$ (1.8gL^{-1}) and yeast extract 18gL^{-1}. The experimental corresponding response was 7.9gL^{-1}. This result agreed the validity and the effectiveness of this model. In addition to establishing optimal fermentation medium composition for

scale up, the RSM was possible to predict productivity under different conditions by means of the contour surfaces and the polynomial model. This is useful not only for the additional knowledge supplied about the process but also for the potential in medium engineering and evaluation under economic constraint of medium composition, yield and productivity (Parra *et al.*, 2005).

This leads to the conclusion that response surface methodology is an excellent means for fermentation medium optimization in the production of single cell protein. From an economic point of view, the most important parameter for screening and optimization of media are time and cost.

In an attempt to approximate industrial conditions for biomass production of *Candida* MCCF101, scale-up was carried out in 5L lab scale fermentor and 100L pilot scale fermentor by using optimized mineral based medium. The maximum biomass production was attained at the late exponential phase of the yeast. In this way, this study achieved a biomass productivity of 16.06gL^{-1} in 5 L lab scale fermentor and 18gL^{-1} in 100 L pilot scale fermentor. The results obtained were encouraging and can be applied to industrial scale conditions.

Molasses as a crude carbon source can be utilized as substrate for the single cell protein production economically. It is an abundant by- product of the sugar industry. The composition of molasses (g 100mL^{-1}) has total sugar 47.85, reducing sugar 28.10, nitrogen 0.60, phosphorus 0.075, calcium 0.744, magnesium 0.138 and ash 8.25 (Bose and Ghose, 1973). Apart from that, molasses also contains water, proteins, vitamins, amino acids,

organic acids, and heavy metals such as iron, zinc, copper, manganese, magnesium, calcium etc. (Roukas, 1998). Many workers have reported the utilization of molasses as carbon source in fermentation processes and in the production of lactic acid, citric acid etc. (Zarowska *et al.*, 2001; Young-Jung *et al.*, 2004). In the present study, molasses concentration of 3.5% was found to be optimal for the growth of the marine yeast *Candida* MCCF 101. Joseph (2009) found that maximum growth of marine yeast *Candida* S 27 was observed at 12mg mL^{-1} total sugars. The higher concentration of molasses did not show any increase in SCP production. Jones *et al.* (1981) found that higher sugar concentration in culture medium probably inhibits fermentation where plasmolysis of yeast cells could occur. Since the common anions and cations occur in sufficient quantities in the crude medium, addition of inorganic sources of potassium, magnesium and calcium did not increase the biomass yield significantly. This supported the result obtained from the previous study (Sarlin, 2005; Sajeevan, 2006) of optimization of media components such as $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $CaCl_2.6H_2O$, KH_2PO_4, which did not significantly influence the biomass production of the same yeast. This may be because of the presence of such ingredients in molasses at comparatively higher level. In the scale up production of 5L fermentor, there was no significant biomass production compared to shake flask experiment.

Finally, a comparison of maximum biomass production obtained in mineral based medium (7.9±0.07gL^{-1} and 16±0.20gL^{-1} in shake flask and laboratory scale fermentor experiment respectively) was made with that in molasses containing medium, and it was found to be 5.8±0.28gL^{-1} and

6.8±0.2gL^{1} in shake flask and laboratory scale fermentor respectively. From this observation maximum biomass production was found to be 2.35 fold in mineral based medium compared to that in molasses based medium.

The biomass production utilizing molasses was low and may be attributed to factors cited below. The crude molasses, although contain high concentrations of glucose, fructose, raffinose and sucrose it may also contain growth inhibitors and heavy metals. Another important factor was the sugar preference of *Candida* MCCF 101. From the shake flask experiments, it was determined that the yeast preferred maltose at 2% compared to other sugars present in molasses.

The industrial production of SCP is greatly affected by the cost of the medium and substrate used, among which the substrate costs are the largest single cost factor involved. Developing an appropriate medium with constituents optimized can significantly save production cost. Simplification of the manufacture and purification of the raw material can save the costs. The energy for compressing air, cooling, sterilizing and drying forms the next most important cost factors. The capital dependent costs are determined by the cost of the apparatus for the process, and the capacity of a plant. The absolute value of the product is governed by the costs involved in production and by the quality of the product.

Table 1 Levels of variables tested in Plackett-Burman Design

Variables (%)	Coded levels	
	-1 (Low)	+1 (High)
Maltose	2	5
NH_4Cl	0.03	0.3
KH_2PO_4	0.2	0.7
$MgSO_4.7H_2O$	0.03	0.08
$CaCl_2.6H_2O$	0.01	0.03
Yeast extract	0.01	0.06

Table 2 Plackett-Burman design matrix for media components

Run	A	B	C	D	E	F	G	H	J	K	L
1	1(5)	-1	1(0.30)	-1	-1(0.2)	-1(0.03)	1(0.03)	1(0.06)	1	-1	1
2	1(5)	1	-1(0.03)	1	-1(0.2)	-1(0.03)	-1(0.01)	1(0.06)	1	1	-1
3	-1(2)	1	1(0.30)	-1	1(0.7)	-1(0.03)	-1(0.01)	-1(0.01)	1	1	1
4	1(5)	-1	1(0.30)	1	-1(0.2)	1(0.08)	-1(0.01)	-1(0.01)	-1	1	1
5	1(5)	1	-1(0.03)	1	1(0.7)	-1(0.03)	1(0.03)	-1(0.01)	-1	-1	1
6	1(5)	1	1(0.30)	-1	-1(0.2)	1(0.08)	-1(0.01)	1(0.06)	-1	-1	-1
7	-1(2)	1	1(0.30)	1	-1(0.2)	1(0.08)	1(0.03)	-1(0.01)	1	-1	-1
8	-1(2)	-1	1(0.30)	1	1(0.7)	-1(0.03)	1(0.03)	1(0.06)	-1	1	-1
9	-1(2)	-1	-1(0.03)	1	1(0.7)	1(0.08)	-1(0.01)	1(0.06)	1	-1	1
10	1(5)	-1	-1(0.03)	-1	1(0.7)	1(0.08)	1(0.03)	-1(0.01)	1	1	-1
11	-1(2)	1	-1(0.03)	-1	-1(0.2)	1(0.08)	1(0.03)	1(0.06)	-1	1	1
12	-1(2)	-1	-1(0.03)	-1	-1(0.2)	-1(0.03)	-1(0.01)	-1(0.01)	-1	-1	-1

(The actual values of factors are given in brackets)

Table 3 Range of variables used for Face Centered Central Composite Design

Variables (%)	Symbol	Coded levels		
		-1 (Low)	0 (Mid)	+1(High)
Maltose	(A)	1	5	9
$MgSO_4$	(B)	0.06	0.13	0.2
Yeast extract	(C)	0.03	1.51	3

Table 4 Plackett-Burman matrix for media components with the corresponding observed and predicted values of biomass production of *Candida* MCCF 101

Run	A	B	C	D	E	F	G	H	J	K	L	Yeast Biomass (gL^{-1})	
												Observed	Predicted
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	3.5	3.66
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	2.96	2.94
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	3.15	3.17
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	3.89	3.54
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	2.98	2.96
6	1	1	1	-1	-1	1	-1	1	-1	-1	-1	3.59	3.61
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	3.17	3.93
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	5	4.88
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	5.5	5.62
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	3.48	3.46
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	4.95	4.79
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	3.01	2.99

A: Maltose, B:Lactose, C:NH_4Cl, D:KNO_3, E:KH_2PO_4, F:$MgSO_4.7H_2O$, G:$CaCl_2.6H_2O$, H:Yeast extract, J:Salinity, K:pH, L:Glycerol

Table 5 Statistical analysis for the Plackett-Burman design

Source		Sum of squares	*df*	Mean square	*F* value	*P* value	
Code	Variables						
Model		8.21	8	1.03	25.39	0.0112	Significant
A	Maltose	2.26	1	2.26	55.95	0.0049	
B	Lactose	0.63	1	0.63	15.59	0.029	
D	KNO_3	0.4	1	0.4	9.89	0.051	
E	KH_2PO_4	0.29	1	0.29	7.05	0.076	
F	$MgSO_4.7H_2O$	1.58	1	1.58	39	0.0083	
G	$CaCl_2$	0.27	1	0.27	6.75	0.08	
H	Yeast extract	2.48	1	2.48	61.22	0.0043	
L	Glycerol	0.31	1	0.31	7.68	0.0695	

Coefficient of determination (R^2) = 0.9854

Adjusted R^2 = 0.9466

Predicted R^2 = 0.7671

Table 6 Percentage of contribution of Plackett-Burman design

Term Intercept	Variables	SumSqr	%Contribution
A	Maltose	2.26201	27.1423
B	Lactose	0.630208	7.56199
C	NH_4Cl	0.003675	0.044097
D	KNO_3	0.399675	4.79578
E	KH_2PO_4	0.285208	3.42227
F	$MgSO_4.7H_2O$	1.57687	18.9212
G	$CaCl_2.6H_2O$	0.273008	3.27588
H	Yeast extract	2.47521	29.7005
J	Dummy	0.0574083	0.688854
K	Dummy	0.0602083	0.722452
L	Glycerol	0.310408	3.72465

Table 7 Face-centered central composite design matrix of the three independent media components along with actual and predicted values of biomass production of *Candida* MCCF 101

No	Maltose (gL^{-1})	$MgSO_4$ (gL^{-1})	Yeast extract (gL^{-1})	Biomass (gL^{-1})	
				Observed	Predicted
1	-1	-1	-1	1.68	1.62
2	1	-1	-1	1.37	1.58
3	-1	1	-1	1.2	1.2
4	1	1	-1	1.5	1.67
5	-1	-1	1	4.1	3.97
6	1	-1	1	6.3	6.34
7	-1	1	1	5.4	5.22
8	1	1	1	8	8.1
9	-1	0	0	5.8	6.17
10	1	0	0	8.1	7.59
11	0	-1	0	7	6.95
12	0	1	0	7.7	7.62
13	0	0	-1	3.2	2.89
14	0	0	1	7.1	7.28
15	0	0	0	7.7	7.77
16	0	0	0	7.65	7.77
17	0	0	0	8	7.77
18	0	0	0	7.27	7.77
19	0	0	0	7.91	7.77
20	0	0	0	7.8	7.77

Table 8 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of Face Centered Central Composite Design

Source	Sum of squares	*df*	Mean square	*F* value	*P* value	
Model	124.58	9	13.84	136.54	< 0.0001	Significant
A	5.03	1	5.03	49.59	< 0.0001	
B	1.12	1	1.12	11.07	0.0077	
C	48.18	1	48.18	475.27	< 0.0001	
A^2	2.16	1	2.16	21.27	0.001	
B^2	0.65	1	0.65	6.39	0.03	
C^2	19.83	1	19.83	195.63	< 0.0001	
AB	0.13	1	0.13	1.26	0.2883	
AC	2.89	1	2.89	28.53	0.0003	
BC	1.4	1	1.4	13.84	0.004	
Residual	1.01	10	0.1			
Lack of Fit	0.69	5	0.14	2.08	0.2197	not significant
Pure Error	0.33	5	0.066			
Cor Total	125.59	19				

Df –Degree of freedom, R^2 =0.9919, Adjusted R^2 = 0.0.9847, Predicted R^2 = 0.9548,

Table 9 Solution predicted by the model for maximum biomass

Solution	Maltose	$MgSO_4$	Yeast Extract	Predicted Biomass	Desirability
1	50.8 (gL^{-1})	1.8 (gL^{-1})	18 (gL^{-1})	8.15 (gL^{-1})	1

Table 10 Production cost of biomass of *Candida* MCCF 101 in mineral based medium

No.	Particulars	Total consumption	Rs/Unit	Rs.×Total consumption	Total cost (in Rs)
1	**Media**				
	Maltose	2500gm	3.4/gm	3.4×2500	8545
	KH_2PO_4	100gm	0.96/gm	0.96×100	96
	$MgSO_4.7H_2O$	90gm	0.22/gm	0.22× 90	19.8
	$CaCl_2.6H_2O$	5gm	1.13/gm	1.13 × 5	5.64
	Yeast extract	900gm	1.64/gm	1.64× 900	1476
2	**Chemiacls**				
	NaOH	600gm	0.38/gm	0.38× 600	230.4
	HCl	151ml	0.28/ml	0.28 ×151	42.28
	Antifoam	9ml	2.75/ml	2.75× 9	24.75
	Total				**10439.87**
3	**Utilities**				
	Fermentor	144 units	8/unit	8 × 144	1152
	Control Unit	36units	"	8 × 36	288
	Boiler	36units	"	8× 36	288
	Air compressor	72units	"	8× 72	576
	Total				**2304**
4	**Labour charge**				
	Manpower	3persons	350/p	350× 3	**1050**
5	**Packing material**				
	Bottles	167 no.	5/bottle	5× 167	833
	Stickers	167 no.	2/stickers	2× 167	334
	Total				**1167**
	Grand Total				**14960.87**
	Total cost/ L			14960.87/50	299.2
	10% of grand total				29.96
					329.16 L^{-1}
	329.16×3(university profit etc.)				987.48
					~988 L^{-1}
	cost/ml			988/1000	0.98 ~ 1ml^{-1}
					Re 1/1ml

Fig. 1 5L lab scale bench top fermentor containing 3L mineral based medium for Biomass production of *Candida* MCCF 101

Fig.2 5L lab scale bench top fermentor containing molasses medium

Fig. 3 Pilot scale 100L fermentor for scale up production of *Candida* MCCF 101

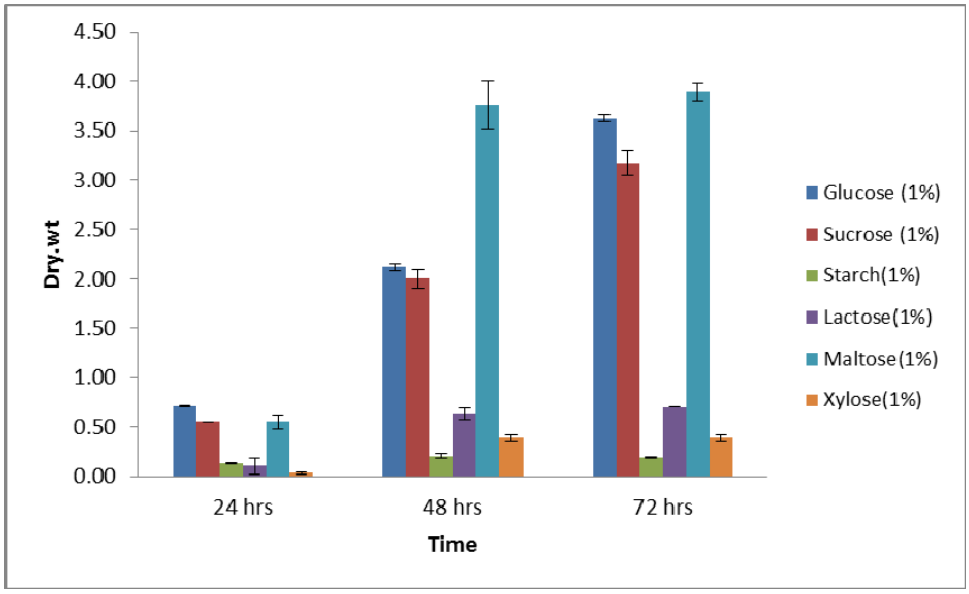


Fig.4 Effect of carbon source on biomass production of *Candida* MCCF 101

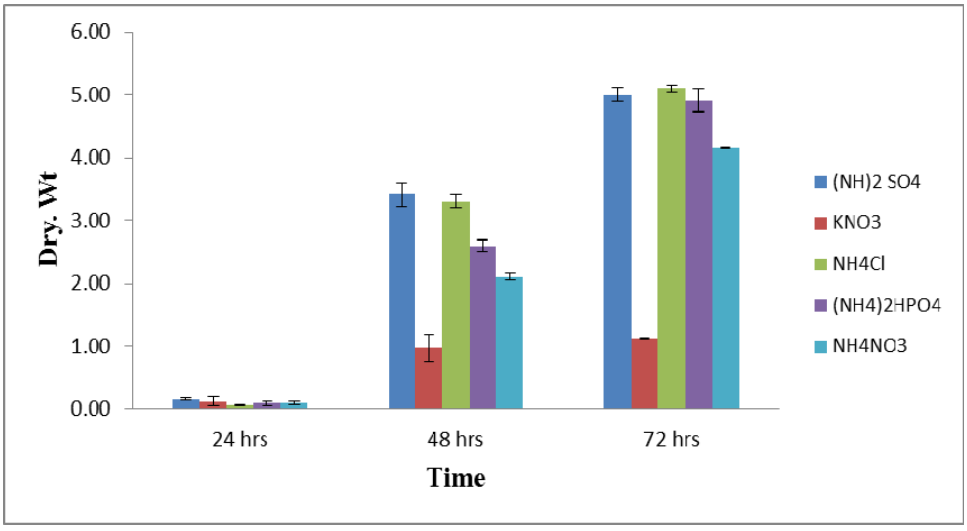


Fig.5 Effect of nitrogen source on biomass production of *Candida* MCCF 101

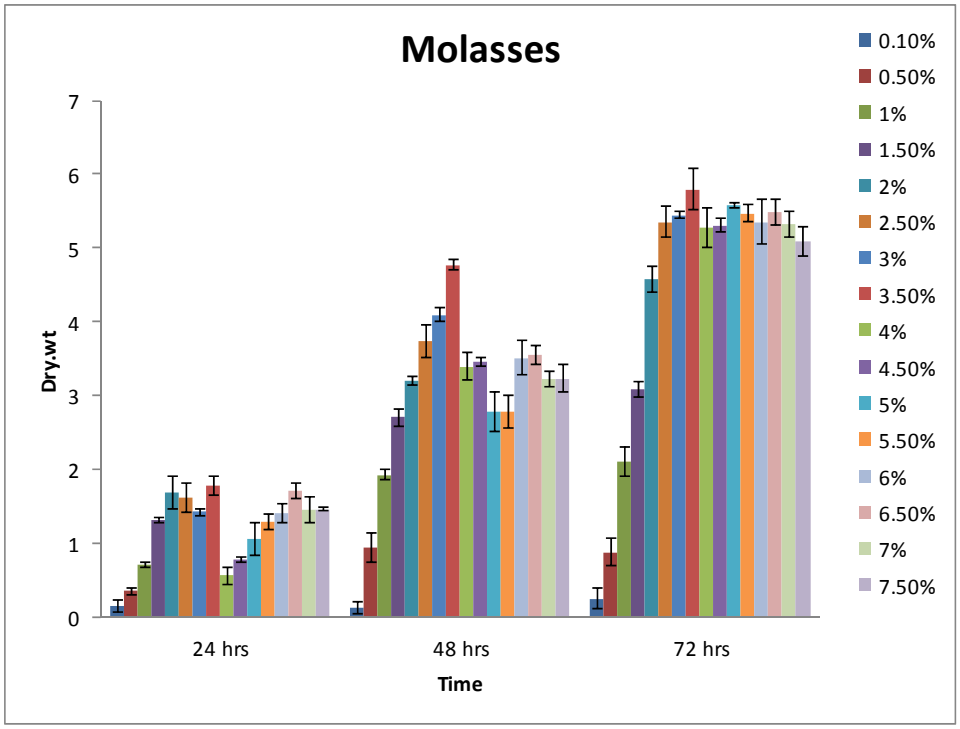
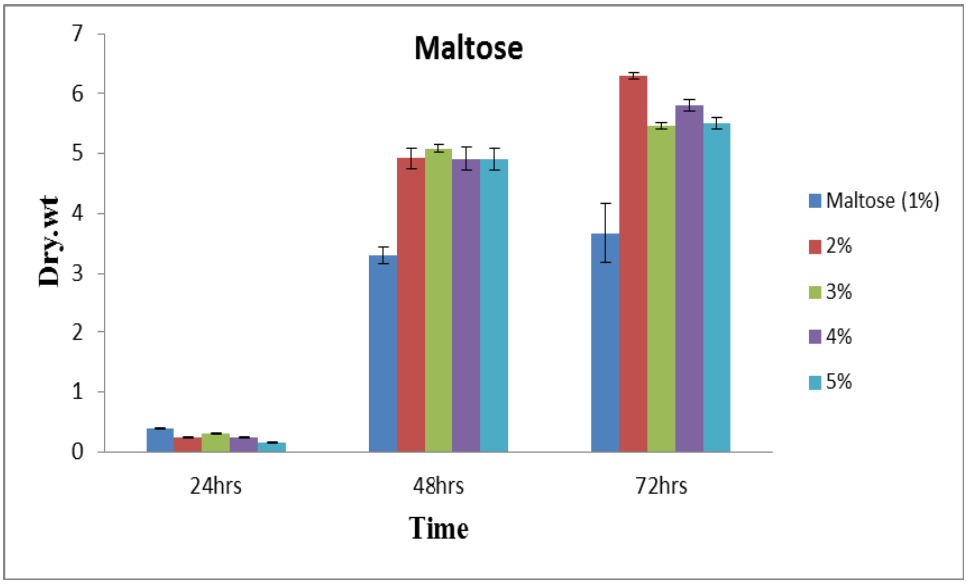
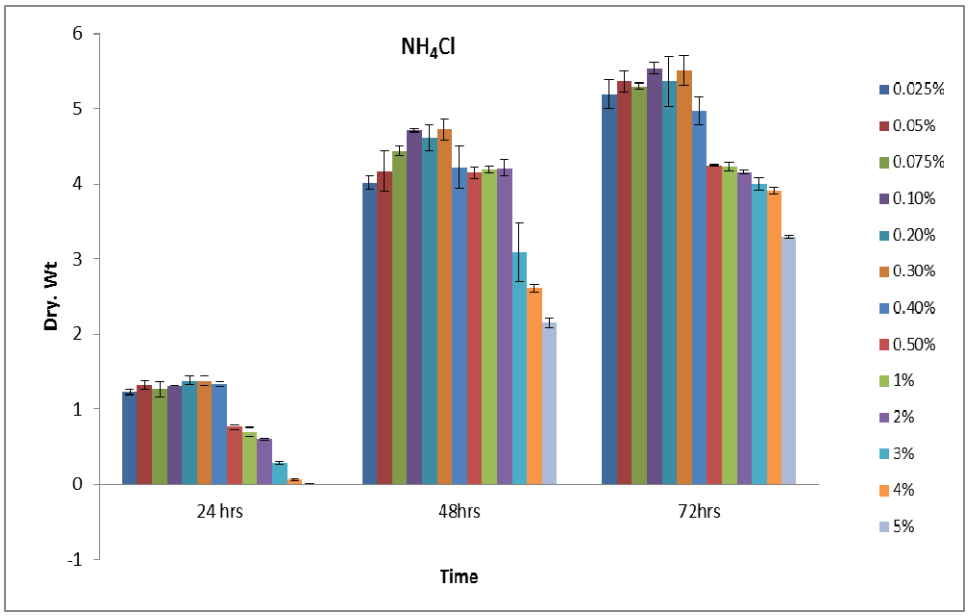


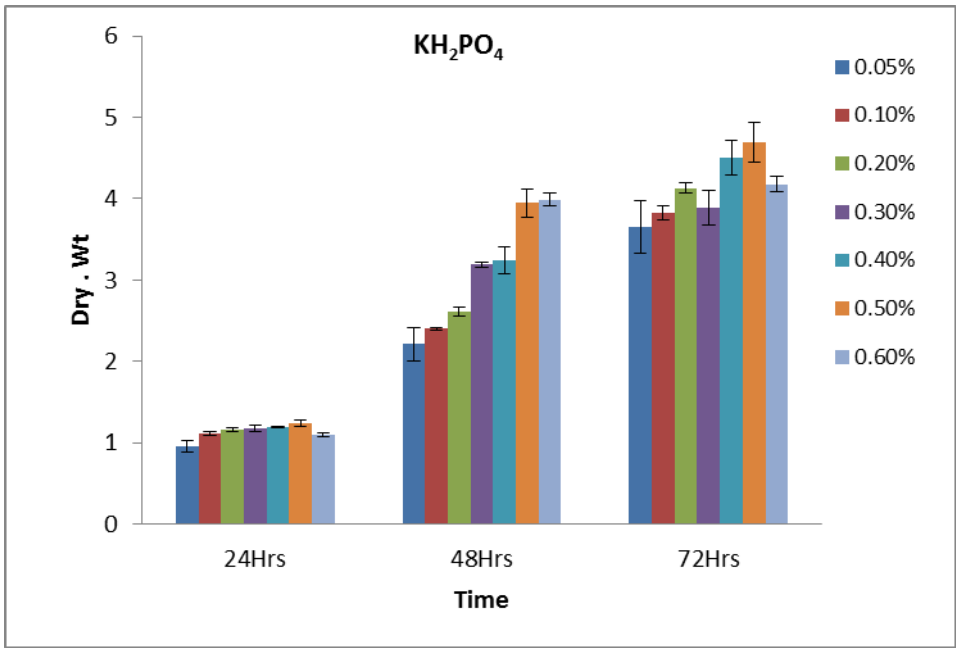
Fig.6 Effect of different concentrations of molasses on biomass production of *Candida* MCCF 101


Maltose
Dry.wt
Time
24hrs
48hrs
72hrs
Maltose (1%)
2%
3%
4%
5%

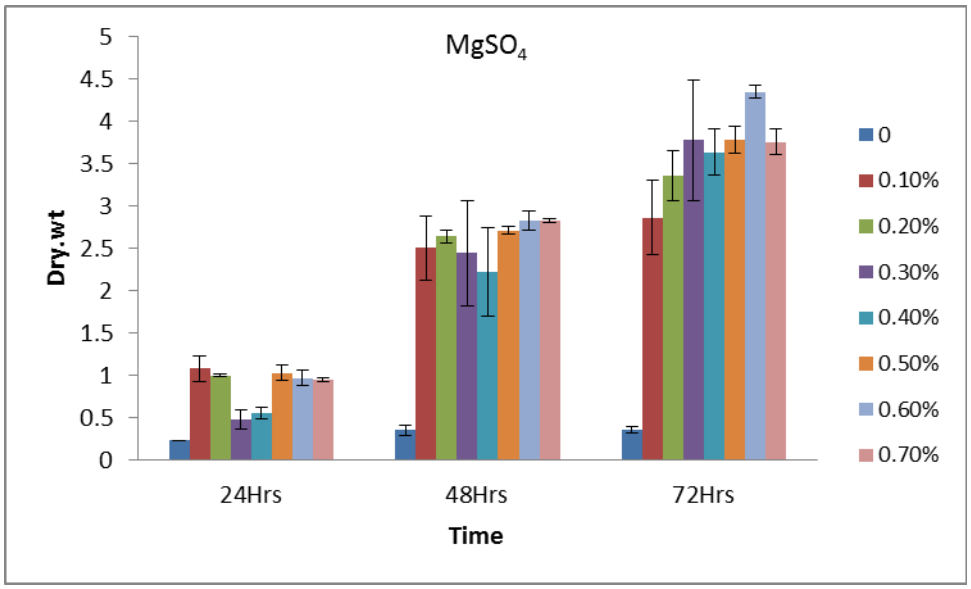
(a)


NH4Cl
Dry. Wt
Time
24 hrs
48hrs
72hrs
0.025%
0.05%
0.075%
0.10%
0.20%
0.30%
0.40%
0.50%
1%
2%
3%
4%
5%

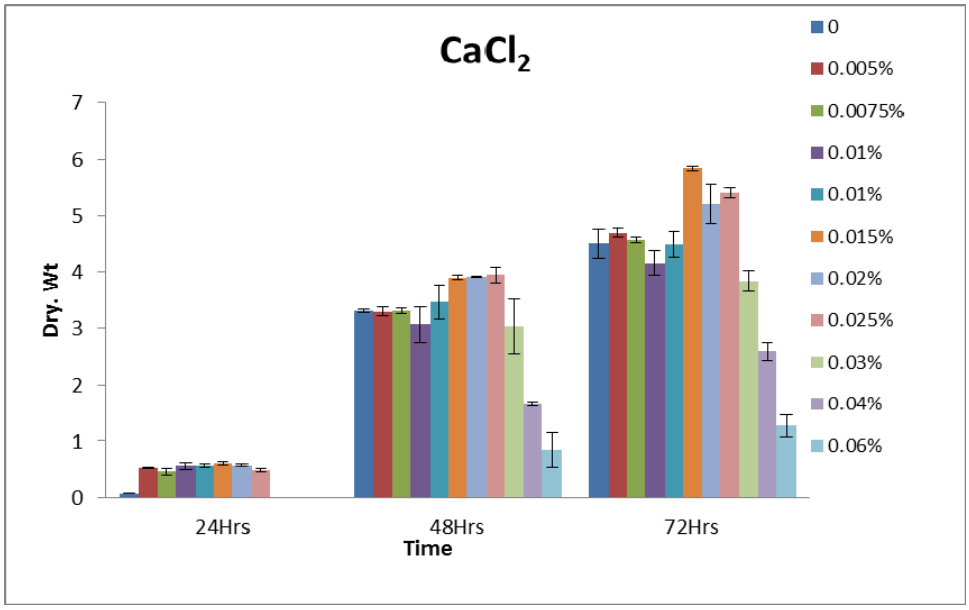
(b)


KH2PO4
Dry . Wt
0
1
2
3
4
5
6
24Hrs
48Hrs
72Hrs
Time
0.05%
0.10%
0.20%
0.30%
0.40%
0.50%
0.60%

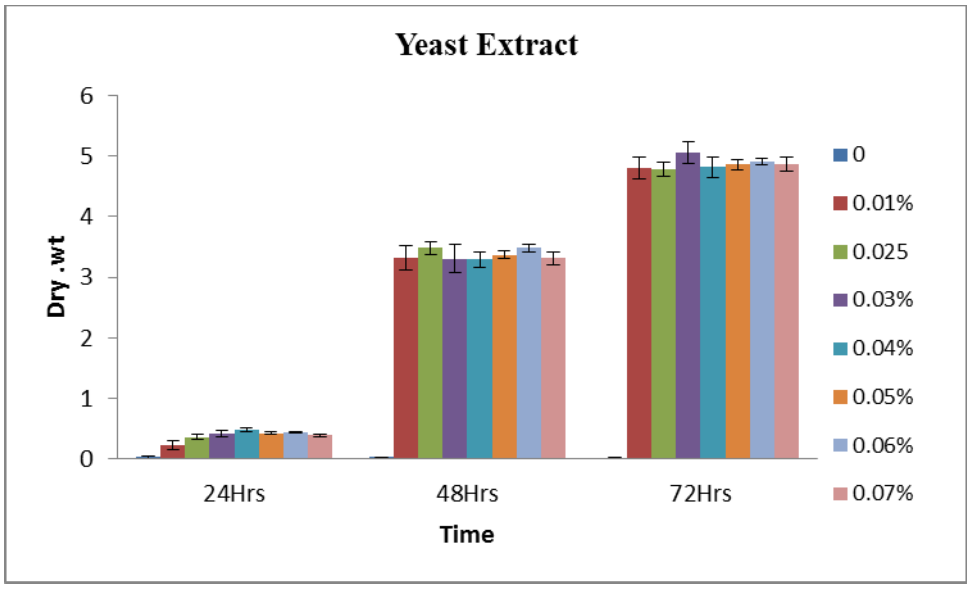
(c)


MgSO4
Dry.wt
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
24Hrs
48Hrs
72Hrs
Time
0
0.10%
0.20%
0.30%
0.40%
0.50%
0.60%
0.70%

(d)

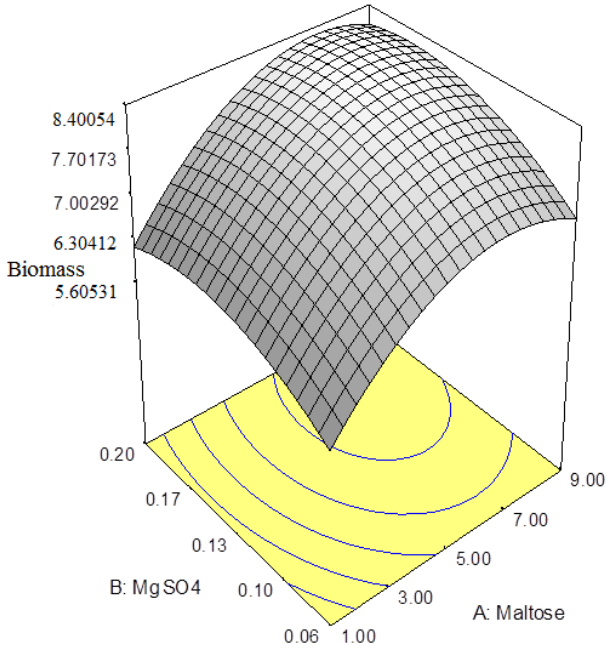


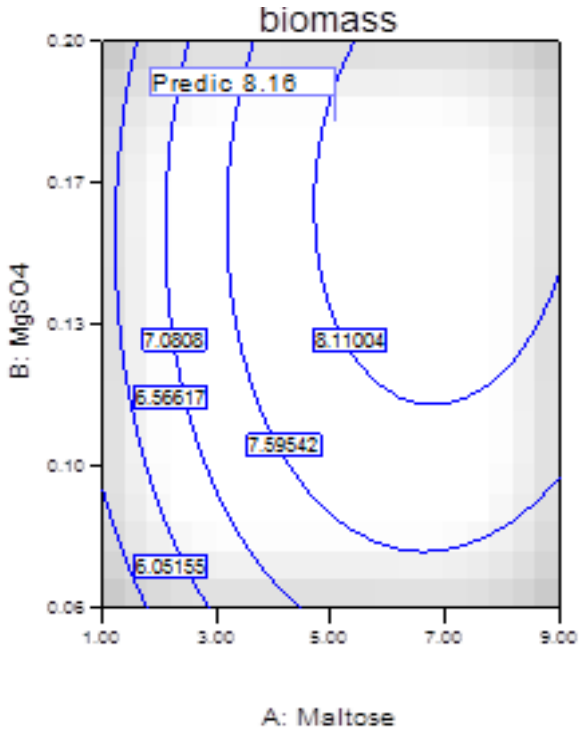
(e)



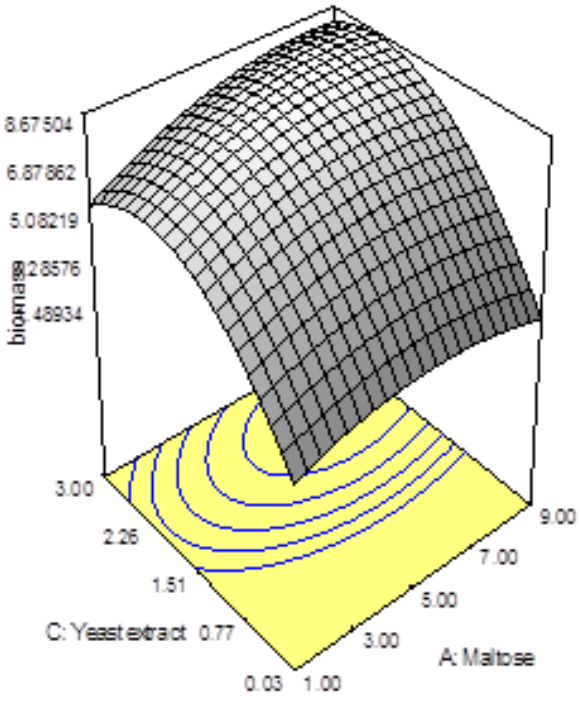
(f)

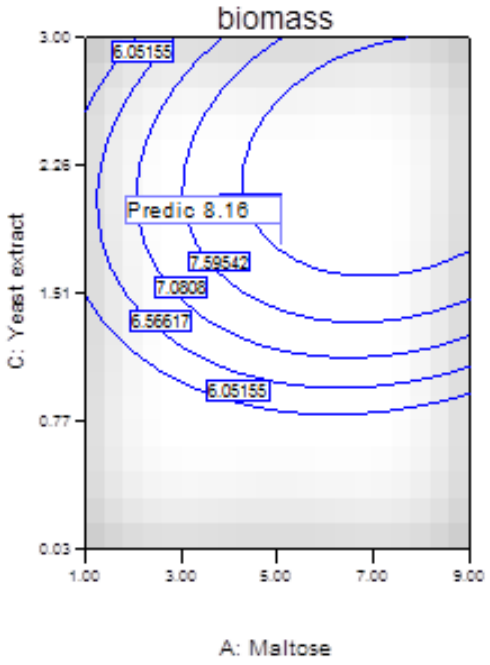
Fig.7 (a-f): One dimensional screening of mineral based media components


8.40054
7.70173
7.00292
6.30412
Biomass
5.60531
0.20
0.17
0.13
0.10
0.06
B: MgSO4
1.00
3.00
5.00
7.00
9.00
A: Maltose

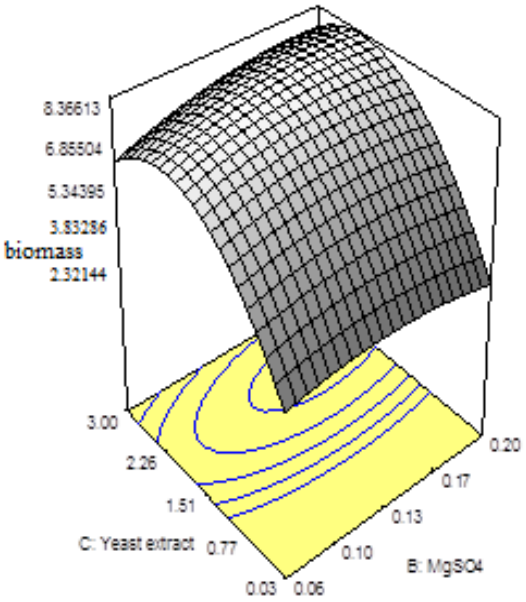

biomass
Predic 8.16
7.0808
8.11004
6.56617
7.59542
6.05155
0.20
0.17
0.13
0.10
0.06
B: MgSO4
1.00
3.00
5.00
7.00
9.00
A: Maltose

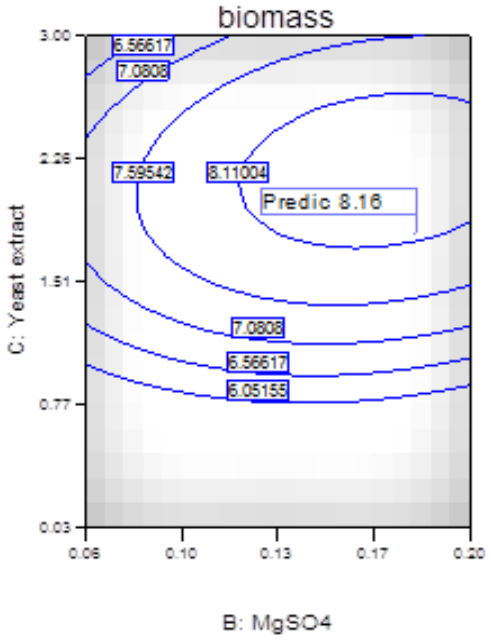
(a)


8.67504
6.87862
5.08219
3.28576
1.48934
biomass
3.00
2.26
1.51
0.77
0.03
C: Yeast extract
1.00
3.00
5.00
7.00
9.00
A: Maltose


biomass
6.05155
Predic 8.16
7.59542
7.0808
6.56617
6.05155
C: Yeast extract
A: Maltose
3.00
2.26
1.51
0.77
0.03
1.00
3.00
5.00
7.00
9.00

(b)





(c)

Fig.8 (a-c) Contour plots and response surface plot for the optimization of biomass production. 3D plot and Contour plot respectively showing the interaction between (a) maltose and $MgSO_4.7H_2O$ (b) maltose and yeast extract (c) $MgSO_4 .7H_2O$ and yeast extract

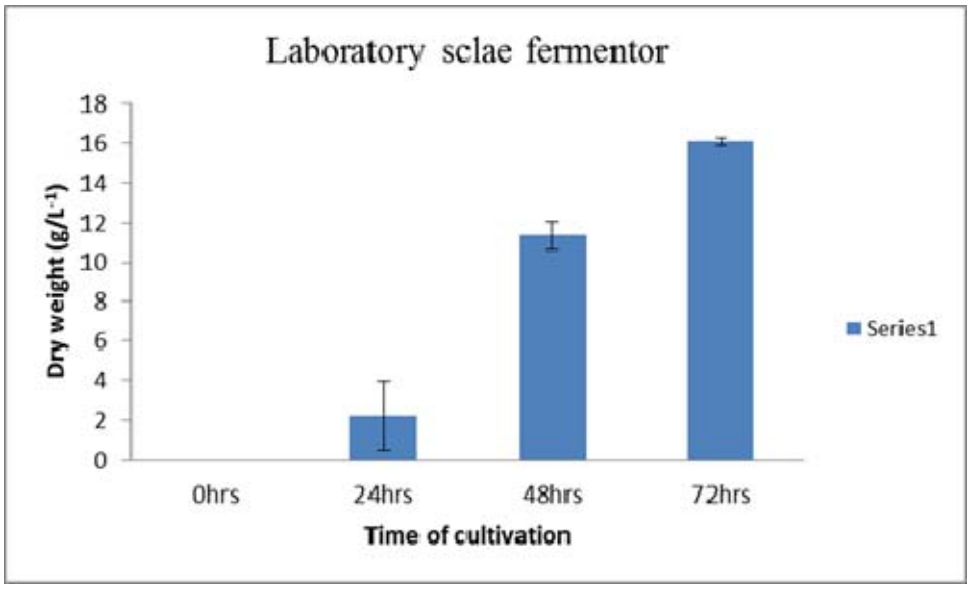


Fig.9 Maximum biomass production using optimized mineral based medium in 5L laboratory scale fermentor

Fig.10 Biomass generation of *Candida* MCCF 101 in 5L bench top laboratory scale fermentor

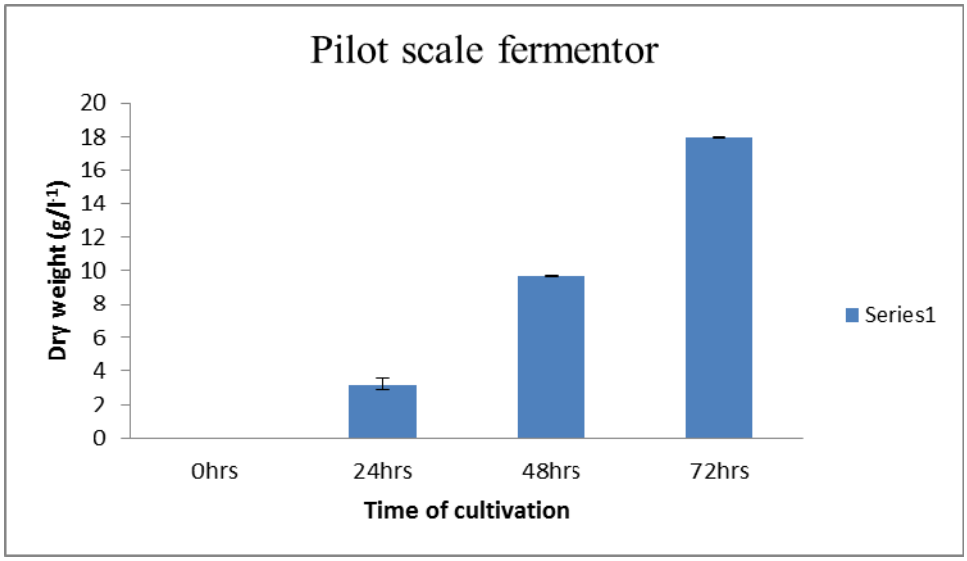


Fig.11 Maximum biomass production using optimized mineral based medium in 100L pilot scale fermentor

Fig.12 Biomass generation in laboratory scale fermentor using molasses based medium

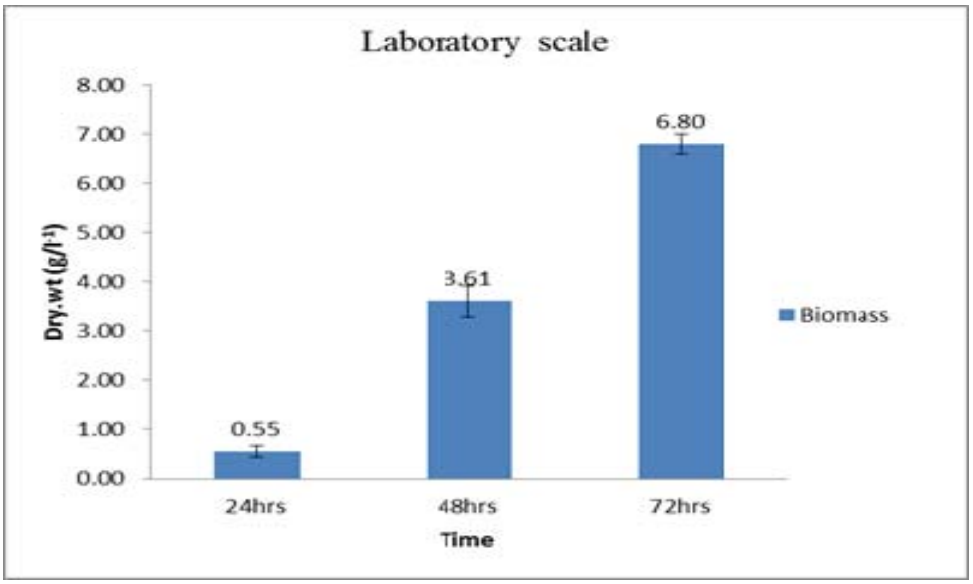


Fig. 13 Biomass production of *Candida* MCCF 101 using molasses based medium in laboratory scale fermentor

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Chapter 5

THE MARINE ISOLATE *CANDIDA* MCCF101 AS DIETARY FEED SUPPLEMENT TO ENHANCE GROWTH, IMMUNOCOMPETENCY AND SURVIVAL OF KOI CARP (*CYPRINUS CARPIO HAEMATOPTERUS*) CHALLENGED WITH *AEROMONAS* SP. MCCB 113

Contents

5.1 Introduction

Rapid growth and disease resistance are the most important concerns in present aquaculture scenario. Intensive aqua farming accompanies several disease problems often due to opportunistic pathogens. High stocking density, high food inputs and other organic loads stimulate the selection and proliferation of opportunistic bacteria (Austin *et al*., 1995). Due to the negative balance of the microbial community in rearing water as well as in fish gut, the aqua culturists often face mass mortality of their stocks. However, with changing scenario farmers are emphasizing on diagnosis and prevention of infection to promote health and production efficiency. The fish health management has now become an integral part of ornamental fish quality assurance programme (Abraham *et al*., 2008). Though the use of antibiotics and chemotherapy remains the method of choice as disease control strategy, the abuse of chemotherapeutics, especially antibiotics has resulted in development of multiple antibiotic resistant bacteria (Alderman and Hastings, 1998; Teuber, 2001). Increased

concern about antibiotic resistant microorganisms has led to the use of alternative dietary supplements such as probiotics and prebiotics to enhance the health and production of cultured fish (Verschuere *et al*., 2000; Merrifield *et al*., 2010a; Ringo *et al*., 2010).

5.1.1 Fish Health Management

Fish Health Management is the concept of proactively regulating the host, pathogen, and environment to maximize the optimal conditions for sustained growth and health. Fish condition indices, based on the length-weight relationship, energy reserves, growth rates, feed conversion, reproduction and survival and relative gonad size (gonadosomatic index) are generally used as indicators of the well-being of fish (Goede and Barton, 1990; Munkittrick, 1992; Adams and Ryon, 1994). Routine maintenance includes standard inspection and/or repair of tanks, net pens, pumps, filters, air supply, or any other life support equipment to ensure the containment and well being of animals. Stocking density, diet, feeding technique, and management procedures all have strong effects on stress levels, subsequent stress tolerance, health, and the presence of aggressive behaviour which turn to affect feedback to one another to further influence welfare (Ashley, 2006). Successful preventive measures in aquaculture center on preventing the introduction of pathogens, maintenance of good water quality, avoidance or reduction of environmental stressors (low dissolved oxygen, temperature control, density control, and removal of metabolic wastes), adequate nutrition, isolation of cultured animals from wild stocks, and immunization (Meyer, 1991).

5.1.2 Fresh water Ornamental fish culture system

The global ornamental fish trade is relatively small but represents a significant segment of the trade in fresh water and marine aquatic products. In 2004, food fish and plants valued at over USD 55 billion were exported, whereas exports of ornamental fish were to the tune of USD 250 million (FAO, 2006).The current trend in aquaculture development is towards subsequent intensification and commercialization of aquatic production. The technique of intensive rearing requires manufactured diet to be given manually or by means of automatic feeders.

Koi carp (*Cyprinus carpio haematopterus*) is a stomach less fish with toothless jaws and reared under the same conditions as gold fish. Digestion takes place in the intestine, which is twice the length of its body. Different enzymes are secreted by pancreas to the intestine. The most important characteristic of koi carp is their colouration, determined by feed and by the genetic makeup of the population. Supplementation can be made entirely with commercially available diet, but this is likely to be expensive which will significantly increase the final product cost. However, it is possible to reserve these commercial diets for periods when the fishes are capable of assimilating them at maximum efficiency. Supplementary diet is chiefly aimed at maintaining weight of fish and the stability of energy reserves in tissues so as to encourage the onset of growth phase in spring. The objective is to produce standard individuals sold at a moderate price with low production costs. High production of carp involve the intensive system of management practices, where antibiotics, drugs and chemicals are used to prevent fish disease caused by environmental stress and other factors.

However, these have been found to be effective only for a short duration besides enhancing the risk of bioaccumulation in the environment.

5.1.3 Pathogenic bacteria in culture system

Disease has now become a primary constraint to the culture of many aquatic species, impeding both economic and social developments and a significant constraint on aquaculture production and trade. The intensive fish culture systems represent highly stressful environments for fish which may suppress the immune response (Kajita *et al*., 1990). Fish grown under these conditions become highly susceptible to diseases. The favorable environment required for pathogenic bacterial growth gets generated in the culture system (Aguirre-Guzman and Felipe, 2000).The bacteria are one of the important causative agents of fish disease (Yesmin *et al*., 2004) and bacterial infections are considered to be a major cause of mortality in fish hatcheries (Grisez and Ollevier, 1995). A disease breaks out when a susceptible fish is exposed to a virulent pathogen under unfavorable environmental circumstances as incidence of a disease is the result of a complex interaction between the fish, the disease agent, and the aquatic environment (Snieszko, 1975). The most frequently encountered bacterial agents associated with fish disease in the tropical environments are *Vibrio* in marine and brackish water systems and motile *Aeromonas* in freshwater environments (Singh *et al*., 1998; Otta *et al*., 2003).

5.1.3.1 *Aeromonas* in fishes

Aeromonas spp. are the primary pathogens of freshwater fish or secondary opportunistic pathogens of compromised or stressed hosts (Jeney and Jeney, 1995).The information on disease caused by *Aeromonads* in

ornamental fishes is comparatively scanty; most of the reports are from cultured food fishes. The genus *Aeromonas* is considered to be the normal inhabitant of the intestinal tract of fishes (Sugita *et al*., 1995; Dugenci and Canadan, 2003; Kozinska, 2007). Two phenotypically distinct groups well known within the genus *Aeromonas* are psychrophilic non motile group and mesophilic motile group. Of the *Aeromonas* spp., *A. hydrophila*, *A. bestiarum, A. salmonicida, A. veronii*, *A. sobria, A. caviae and A. jandaei* have been reported as pathogens of various fish species (Popoff, 1984; Austin and Austin, 1999; Nielsen *et al*., 2001; Kozinska *et al*., 2002; Rahman *et al*., 2002; Dugenci and Canadan, 2003; Shome *et al*., 2005; Wahli *et al*., 2005, Sreedharan and Singh, 2011).

Motile *Aeromonas* are associated with more than one disease manifested through several clinical signs like fin rot and tail rot, ulceration, exophthalmia, and abdominal distention. It is responsible for Motile Aeromonad Septicemia (MAS), Bacterial Hemorrhagic Septicemia (BHS) and is associated with epizootic ulcerative syndrome in numerous fresh water fishes (Rahman *et al*., 2004).

5.1.4 Prevention of disease

Disease is now a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries (Bondad-Reantaso *et al*., 2005). It has long been recognized that poor water quality, environmental and physiological stressors, and poor nutrition are the primary causes of disease outbreaks. Prevention is an important component of a fish health management program. Disease prevention and treatment strategies such as vaccinations and drugs are currently limited in large-scale aquaculture due to regulatory constraints or

inconvenient administration protocols. The aquaculture industry began to focus on the prevention of disease rather than treatment (Baulny *et al*., 1996) with chemotherapeutants and antibiotics, which have been criticized for their negative side effects. In recent years there has been heightened research in developing dietary supplementation strategies in which various health-promoting compounds have been evaluated.

5.1.4.1 Antibiotics

Traditionally antibiotics have been used in aquaculture for the prevention and treatment of bacterial disease. The disadvantage of this therapy in aquaculture poses threats such as development of bacterial strains that are resistant to antibiotic treatment. Certain antibiotics have also been shown to suppress the immune system of the cultured species. This has led to the ban of subtherapeutic antibiotics in some countries. It must be remembered that widespread use of antimicrobials is not a substitute for efficient management or good husbandry. Alternative methods of disease control should be used to reduce antimicrobial use.

5.1.4.2 Probiotics

Probiotics are dietary supplements and live microorganisms consisting potentially beneficial live bacteria or yeast. This health benefit is established by affecting the intestinal microbial balance of the host organism (Wang and Xu, 2006). The addition to or altering of the intestinal microbiota has been done to achieve such positive effects as enhanced growth, digestion, immunity and disease resistance. Probiotics are usually selected to control specific pathogens through competitive exclusion or enhancement of fishes' immune system. This means that the list of

probiotics is steadily increasing. Examples of probiotics include Gram-positive bacteria such as *Bacillus* sp, *Carnobacterium inhibens* K1 and *Lactobacillus* sp. The restrictions on application of probiotics in aquaculture include the costs as well as the insufficient evaluation of the biological consequences and the potential influence on natural microbial diversity (Hoffmann, 2009).

5.1.4.3 Prebiotics

These are non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the intestinal tract, and thus improve host health. The main advantage of prebiotics over probiotics is that they are natural feed ingredients and thus regulatory control over dietary supplementation should be limited. The classic examples of prebiotics are fructooligosaccharide (FOS), galacto-oligosaccharides (GOS), trans galacto-oligosaccharides (TOS) and mannan oligosaccharides (MOS). The inclusion of prebiotics in the diet has been reported to increase the uptake of glucose and the bioavailability of trace elements most likely by decreasing the pH of the intestinal tract. Moreover effects on volatile fatty acids (VFAs) have been observed (Hoffmann, 2009).

5.1.4.4 Synbiotics

Synbiotics refer to nutritional supplements combining probiotics and prebiotics to form a symbiotic relationship. This is a new concept for aquaculture which needs further evaluation and more in-depth research to fully characterize the effects in aquatic organisms. Improving general health and performance in aquaculture by stimulating the growth of specific

microbes in the intestinal tract or directly stimulating the immune system basically is a good and rational strategy. Nevertheless, the basic requirements of a diet to supply adequate quantities of essential and non-essential nutrients for various organisms must not be neglected. The stimulation of growth of intestinal bacteria by pro or prebiotics involves the supply of sufficient amounts of other nutrients to enable the multiplication of these microbes. If the supply of basic nutrients cannot be guaranteed by the diet or the animal itself, the beneficial effects of pro- and/or prebiotics will be reduced or even unverifiable (Hoffmann, 2009).

5.1.4.5 Immunostimulants

The immunostimulants are used to boost the fish or crustacean immune system. It may be used to elevate the non-specific defense mechanisms, to reduce stress and mortalities, and to maintain health of the cultured organisms (Raa, 2000). These includes vitamin-mineral mixes, vitamin C, products containing glucan, gut-probiotics, extracts of other natural products and herbal extracts mixed with feed by binding agent. As research on the use of immunostimulants for the prevention of fish diseases progresses, several preparations and regimes have become more promising (Jeney and Jeney, 2002). Use of immunostimulants in aqua feed is considered to be safe and effective against various pathogens. Many of the substances tested have immunostimulating properties in fish and have been shown as effective in raising non-specific defense mechanisms, and specific immune response and protection against fish diseases (Nikl *et al*., 1991; Anderson, 1992). These include neutrophil activation, the production of peroxidase, oxidative radicals and instigation of other inflammatory factors. Yeast contains various immunostimulating compounds such as β-glucans, nucleic acids,

and oligosaccharides, and it has the capability to enhance the growth of various fish species (Oliva-Teles and Goncalves, 2001; Lara-Flores *et al*., 2003; Abdel-Tawwab *et al*., 2008) and the immunostimulant properties (Ortuno *et al*., 2002; Rodríguez *et al*., 2003; Cuesta *et al*., 2004; Esteban *et al*., 2004; Li and Gatlin, 2005) have been well studied.

5.1.4.5.1 *β-glucans*

β- 1, 3 - glucans, which are most commonly found in the cell walls of yeast, is generally considered as the main factor for its immune stimulating properties (Gannam and Schrock, 2001). The cell wall of yeast cells is mainly composed of mannoproteins and β-linked glucans and has β-1, 3- and β-1, 6-linked glucose and a fibrillar or brush-like outer layer composed predominantly of mannoproteins (Ueda and Tanaka, 2000). β- glucans have been used as immunostimulants to enhance the defense potential of fish and shellfish against bacterial or viral infection (Sakai, 1999) and is becoming affordable for the aqua feed industry. Yeast glucan also has adjuvant effects on marine animals and the abilities to enhance the lysozyme activity, complement activity and bacteria-killing activity of macrophages of marine animals and the production of superoxide by macrophages or hemocytes in some marine animals (Sakai, 1999).

5.1.4.5.2 *Nucleotides*

Nucleotides are biological compounds with low molecular weight that play key roles in essential physiological and biochemical functions including encoding and deciphering genetic information, mediating energy metabolism and cell signaling as well as serving as components of coenzymes, allosteric effectors and cellular agonists. It is well documented

that nucleotides improve growth performance, increase stress tolerance, affect serum biochemical parameters, and modulate immune responses of fish and crustaceans (Yousefi *et al*., 2012). It was thought that all organisms could supply sufficient amounts of nucleotides to meet their physiological demands. However, under certain conditions, including rapid growth, limited food supply, stress, immunological challenges and some others, dietary nucleotides turn to conditionally essential nutrients. Balanced formulations of purified dietary nucleotides modulate innate and adaptive immune response as reported in numerous scientific publications (Burrells *et al*., 2001; Sakai *et al*., 2001; Dalmo, 2005). Prolonged administration of medication or immunostimulants often leads to undesirable side effects on growth and disease resistance.

5.1.4.6 Single Cell Protein (SCP)

Single cell proteins (SCP) include micro algae, bacteria and yeast, and are alternative non-conventional protein sources that are frequently used as feed ingredients for fish, due to the nutritional value such as proteins, B-vitamins, pigments and complex carbohydrates, such as glucans (Sanderson and Jolly, 1994; Tacon, 1994). Among SCP, yeasts have been the most widely used within aquafeeds (Tacon, 1994). Yeast single-cell proteins (SCPs) are playing a greater role in the evolution of aquaculture diets. Some yeast, like *Candida* sp. and *Saccharomyces cerevisiae*, are also believed to have immunostimulatory properties by virtue of their complex carbohydrate components and nucleic acid content (Anderson *et al*., 1995). With excellent nutrient profiles and capacity to be mass produced economically, SCPs have been added to aquaculture diets as partial replacement of fishmeal (Lim *et al*., 2005).

5.1.5 Yeast as feed supplement

The use of yeast cell walls or even whole yeast in fish farms would be of interest because different yeasts have been used successfully in fish feed as a protein source, substituting expensive fish meal protein (Ortuno *et al*., 2002). Some yeasts such as *Saccharomyces cerevisiae, Candida utilis, Candida tropicalis* and the species of genera *Hansenula, Pichia* and *Torulopsis* can be used for single cell protein. Their protein contents account for up to 50% of the dry cell weight. Moreover, they can also supply the feed with the B-complex group vitamins, minerals and other components, which could stimulate the disease resistance of marine animals (Zhenming *et al*., 2006). The digestion rate of single cell protein of yeast cells is generally above 80% (Ravindra, 2000). Brown *et al*. (1996) found that the marine yeasts *Debaromyces hansenii* ACM 4784, *Dipodascus capitatus* ACM 4779 and *Dipodascus* sp. ACM 4780 contained 23%, 32%, and 36% of crude protein, respectively, while terrestrial *Candida utilis* ACM 4774 contained 42% of crude protein. They concluded that high protein content, high levels of carbohydrate and good amino acid composition characterized all the marine yeasts, while high levels of saturated fats characterized only few marine yeasts. Yeast contains various immunostimulating compounds such as β-glucans, nucleic acids as well as mannan oligosaccharides, and it has the capability to enhance immune responses (Siwicki *et al*., 1994; Anderson *et al*., 1995; Ortuno *et al*., 2002) as well as growth (Oliva-Teles and Goncalves, 2001; Lara-Flores *et al*., 2003; Li and Gatlin III, 2003; 2004; 2005) of various fish species. However, the administration of yeast has been recognized to have important effects on immunostimulant functions (Sakai, 1999). Moreover, these types of naturally available immunostimulants recorded less side

effects and were more cost effective than the commercial products. The administration of yeast through diet may serve as dietary supplements to improve fish growth and immune response. These natural feed additives positively influenced the non-specific immune responses of many aquaculture species (Siwicki *et al*., 1994; Anderson *et al*., 1995; Thanardkit *et al*., 2002). Possible use of yeast in fish diets has many advantages. Firstly, they can be produced rapidly, easily and inexpensively and, at the same time, they are very stable and can be recycled from other industries. They are natural substances and hence no negative effects may be expected either to the animals or to the environment. Moreover, there is no need to isolate their components, which consists mainly of cell wall sugars (β-glucans, mannoproteins and chitin); all of which are well-proven immunostimulant compounds (Tewary and Patra, 2011).The dietary intake of whole yeast cells has also been demonstrated their immunostimulant properties enhancing leukocyte phagocytosis and respiratory burst (Cuesta *et al*., 2007).

5.1.6 Health Assessment

The application of haematological and serological techniques have proved valuable for fishery biologists in assessing the health of fish and monitoring stress responses either due to fluctuations in environmental condition or due to sub lethal concentration of pollutants. Blood parameters are useful and sensitive for the diagnosis of diseases and monitoring of the physiological status of fish exposed to toxicants, which has been shown by Adhikari *et al*. (2004).

5.1.6.1 Haematology

Haematological parameters (such as number of erythrocytes and amount of haemoglobin) are regarded as valuable tools for assessing fish health (Houston, 1997; Asadi *et al*., 2006; Hoseinifar *et al*., 2011) and to assess conditions required to optimize growth, feed conversion, reproduction and survival. The major investigations centered are on red blood cell (RBC) count, haemoglobin concentration (Hb), packed cell volume (PCV), white blood cell (WBC) count, mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV). They are influenced by intrinsic and extrinsic factors (Nespolo and Rosenmann, 2002; Rios *et al*., 2002).

5.1.6.2 Serology

Serum biochemical parameters are useful indices for monitoring the health and physiological condition of aquatic vertebrates (Shi *et al*., 2006b; Fanouraki *et al*., 2007; Hoseinifar *et al*., 2010). Serology deals with the constituents in the fluid part of blood such as protein, enzymes, minerals, carbohydrates, pigments, hormones, immune bodies etc (Kapila, 1999). The serum proteins, composed of a non homogeneous mixture, may be classified according to various physical and chemical properties. Basically the serum proteins are divided into two major fractions – albumin and globulin. Albumin and some of the globulins are synthesized in the liver. The proteins in plasma and sera are chiefly involved in nutrition, water distribution, acid-base balance, transport mechanism, immunity and enzymatic responses to specific metabolic needs. Serum protein concentrations can be used to monitor disease progress and general physiological status, as total protein levels tend to drop in diseased states. Sequential total protein analyses provide quantitative evidence of disease progression (Searcy *et al.*, 1964).

Therefore the present study was conducted to determine the growth performance, feed utilization and non-specific immune responses of koi carp fed with the marine yeast isolate *Candida* MCCF 101 and challenged with *Aeromonas* sp. MCCB 113.

5.2 Materials and Methods

5.2.1 Preparation of yeast biomass

Yeast biomass was generated in pilot scale fermentor containing mineral based medium (maltose -50.8gL^{-1}, $MgSO_4.7H_2O$-1.8gL^{-1}, and yeast extract-18gL^{-1}) with pH 6.51 and incubated at 26.3^oC for 72 hrs. One portion of the cell biomass was harvested at exponential phase as live yeast and the other portion steamed at 10 lb for 10 min in the fermentor itself for inactivation.

5.2.2 Experimental diet

Experimental diets were prepared by incorporating different concentrations of yeast biomass to get the final count of 10^6, 10^7, 10^8 and 10^9cfu g^{-1} (both live and inactivated) standard fish diet. This was done initially by incorporating yeast to the binder, 'Stick On' India and coating on to the feed. After air drying for few hours, the pellets were kept in desiccator overnight for complete drying. The experimental diets were stored in plastic bags at 4^oC for further use.

5.2.3 Experimental Animals

Koi carp (*Cyprinus carpio haematopterus*), the fresh water ornamental fish, obtained from a fish hatchery located at Thrissur, Kerala, India was used for the study. Fishes weighing ~1g ± 0.2g were acclimatized in a covered aquarium tank containing fresh water (Fig.1a) over a period of two weeks

until feed consumption and general behavior became normal. Water temperature ranged from 27^{o} to 29^{o}C, dissolved oxygen concentrations from 4.3 to 6.7mgL^{-1}, pH from 7.2 to 8.0, and unionized ammonia concentration from 0.04 to 0.14mgL^{-1}.After the period of acclimatization, the fishes were transferred to the experimental tanks (Fig.1b-c) and were allowed to acclimatize for another week.

5.2.4 Experimental design

After acclimatization, fishes were randomly divided in to nine groups. One was kept as control and the other eight groups included treatment with live and inactivated yeast at concentrations 10^{6}, 10^{7}, 10^{8} and 10^{9}cfu g^{-1}. Each group consisted of three replicates of 10 animals in each tank, i.e., n= 30 for each group. There were a total of 270 animals in the experiment tanks under the same rearing conditions. Each aquarium was supplied with compressed air through air sparger using aquarium air pumps. Fish wastes settled at the bottom of the tanks were siphoned out daily along with three quarters of the aquarium water, which was replaced by aerated water from the storage tank. The basic physico-chemical parameters of water viz. temperature, dissolved oxygen, NH_3-N, NO_2-N and NO_3-N were monitored daily following standard procedures (APHA, 1995) and maintained at optimal levels. Stringed Bed Suspended Bioreactor (SBSBR) (Kumar *et al*., 2009) was maintained in all the tanks to manage ammonia level around 0.1ppm.

5.2.5 Feeding regime

Control groups were fed with commercial feed without supplementation of yeast. Remaining groups were fed with varying concentrations of inactivated and live yeast preparations such as 10^{6}, 10^{7}, 10^{8} and 10^{9} cfu g^{-1}.

The yeast incorporated diets were initially fed at 10% of body weight for 4 weeks, subsequently reduced to 5% during the remaining weeks. Each diet was fed twice daily for a period of three months. Uneaten pellets were siphoned out of the tanks. Fishes in each tank were weighed once every 10 days. All fishes were individually weighed using analytical balance and the feed ratio was adjusted accordingly. Mortality was recorded daily and dead fishes were removed.

5.2.6 Growth performance

Growth performance was assessed in terms of mean final weight gain, percentage of weight gain, specific growth rate (SGR), Feed efficiency (FE), feed conversion ratio (FCR) total protein intake (PI) and protein efficiency ratio (PER). These were calculated as follows:

Weight gain=W_2 (g) −W_1 (g)

Percentage of weight gain = wt. gain/ W_1 × 100

Specific growth rate (SGR) =100 (ln W_2−ln W_1)/T;

(Where W_1 and W_2 are the initial and final weight, respectively, and T is the number of days in the feeding period)

Feed efficiency (FE) = weight gain (g)/ total feed intake (g) × 100

Feed conversion ratio (FCR) =feed intake (g)/weight gain (g)

Total protein intake (PI) = feed intake (g) × protein in feed (g)

Protein efficiency ratio (PER) =weight gain (g)/protein intake (g)

5.2.7 Physiological parameters

Fishes were not fed for 24 hrs prior to blood sampling and were anaesthetized with clove oil in ethanol at ratio of 1:10 (v/v) and added to water to get a final strength of 80ppm (Fig.2 a-b). The point at which the fish lost sensitivity to touch used for blood collection. Blood was collected by tail ablation. Using a haematocrit tube, blood was taken from caudal vein (Fig.3 a-d). The extracted blood was divided in two sets of Eppendorf tubes. One set contained a pinch of EDTA (ethylene diamine tetraacetic acid) used as an anticoagulant for haematological analysis (Hb, RBC, WBC and PCV). The second set was left to clot at 4°C and centrifuged at 5000 rpm for 5 min at room temperature. The collected serum was stored at −20°C for further assays (glucose, albumin, globulin and protein). Blood samples pooled from a random sample of fish in each experimental tank was used.

Haemoglobin (Hb) level was determined colorimetrically by measuring the formation of cyanmethaemoglobin using a commercial kit. In this method the ferrous ions (Fe^{2+}) of haemoglobin are oxidized to ferric state (Fe^{3+}) by potassium ferricyanide to form methaemoglobin. The methaemoglobin then reacts with cyanide ions from potassium cyanide to form cyanmethaemoglobin which can be measured colorimetrically. Red blood cells (RBCs) and White blood cells (WBC) were counted under a light microscope using a Neubauer haemocytometer following the method described by Praful and Darshan (2003). Packed Cell Volume (PCV) was determined by microhaematocrit method. Microhaematocrit method employs small capillary tube of 8cm length with a uniform pore size of 1mm diameter. PCV as cell volume percent was measured directly on a

microhaematocrit reader associated with the centrifuge. Glucose was determined colorimetrically according to Sasaki *et al*. (1972). Serum total protein content was estimated by the method of Lowery *et al*. (1951). Total lipid content was determined colorimetrically according to Barnes and Blackstock (1973). Albumin and globulin were determined colorimetrically according to Bartholomew *et al*. (1966).

5.2.8 Challenging with *Aeromonas sp.* MCCB 113

5.2.8.1 Bacterial culture

Aeromonas sp. MCCB 113 originally isolated from diseased Koi carp and characterized (Sreedharan, 2008) was obtained from the culture collection of NCAAH. The pathogenic isolate was cultured two times successively in brain heart infusion (BHI) agar plate and transferred to BHI agar slants, incubated at 28^{o}C overnight, and harvested in 0.5% saline. The cell density was adjusted to absorbance 1.0 at Abs_{600nm} and serially diluted to get 10^4 to 10^8 cells mL^{-1}. The viable counts of the dilutions were determined by spread plate technique and the colonies were counted after 24 hr at 28^{o}C on nutrient agar plate.

5.2.8.2 Determination of LD_{50} for *Aeromonas* sp. MCCB 113

The dosage was determined by LD_{50} of the bacterium by intra-peritoneal (IP) injection with different doses of *Aeromonas* sp. MCCB 113 (Fig.4 a, b, c, d). Seven fishes from five groups were administered with 0.1 ml of saline suspension of graded dosage of *Aeromonas* sp. MCCB 113 such as 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 cfu mL^{-1}. Control group was injected with 0.1ml saline. Fishes were observed for a week for external signs of disease, and mortality rates were recorded. Specific mortality of

fish was confirmed by the re-isolation of the pathogen from the fish. The LD_{50} was calculated following Reed and Muench (1938).

5.2.8.3 Challenge experiment

At the end of the study, fishes were challenged with the pathogen *Aeromonas* sp. MCCB 113. The fishes in each experimental group were injected intra-peritoneally (IP) with 0.1mL (1×10^7cfu mL^{-1}) of the *Aeromonas* sp. MCCB 113. All groups were kept under observation for 10 days to record clinical signs of mortality. The cause of death was confirmed by re-isolating the organism from kidney of dead fishes (10% of dead fishes were used for reisolation) using *Aeromonas* isolation agar. Percentage survival was calculated employing the following formula:

$$\% \text{ survival} = \frac{\text{No.of surviving fish after challenge}}{\text{No.of fish injected with the pathogen}} \times 100$$

5.2.8.4 Identification of re-isolated bacterial pathogen

5.2.8.4.1 *Phenotypic characterization*

The re-isolated pathogen was identified as *Aeromonas* sp. based on the phenotypic characteristics such as Gram's stain, motility, oxidation fermentation reaction, Kovac's oxidase, resistance to O/129, utilization of DL-lactate and acid production from sucrose, D-cellobiose and salicine.

5.2.8.4.2 *Motility assay*

Motility was tested in soft agar medium having beef extract (5g), peptone 5(g), agar (3g) and distilled water (1L) with pH 7.2±0.1. The

medium was prepared in tubes in 3ml aliquots and autoclaved at 15lbs for 15min and stab inoculated. Rhizoidal growth from the line of inoculation towards the peripheral area was considered as the sign of motility.

5.2.8.4.3 *Oxidation Fermentation reaction*

Marine Oxidation fermentation (MOF) medium (Himedia) was employed for the determination of oxidation fermentation reaction. The pH indicator in the medium was phenol red. A quantity of 2.2g MOF medium was transferred to 100ml distilled water, solidified using 1.5g agar and sterilized at 15lbs for 15 min. To the molten medium 1% glucose was added and transferred 4ml aliquots aseptically into sterile tubes and autoclaved at 10lbs for 10 minutes and made into slants with long butt. The tubes were stabbed and streaked and incubated at 28±0.4^{o}C. When glucose was utilized, acid production changed the color of the medium from pink to yellow. Pink coloration at the butt and yellow color in the slope indicated an oxidative reaction, whereas the whole tube turning yellow indicated a fermentative reaction.

5.2.8.4.4 *Kovac's Oxidase test (Cytochrome oxidase activity)*

According to the method recommended by Kovacs (1956) the organisms were freshly grown on nutrient agar slants. A platinum loop was used to pick a bit of inoculum and made a compact smear on a filter paper moistened with 1% solution of tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive result was recorded when the smear turned to violet within 10 seconds, indicating the formation of indophenol.

5.2.8.4.5 ***Sensitivity to vibriostatic compound O/129 (2, 4-diamino-6, 7-di-iso propyl pteridine phosphate)***

The nutrient agar plates were prepared and swabbed with the suspension of the test bacterial culture. Discs of O/129 (6mm diameter of Whatman filter paper containing 150µg ml^{-1} of the compound) were placed on the plate with appropriate spacing. The cultures sensitive to the pteridine compound developed clearing zones around the disc. *Vibrio* and *Photobacterium* are sensitive to the vibriostatic compound while *Aeromonas* and *Leucibacterum* resistant.

5.2.8.4.6 ***Utilization of DL-lactate***

Utilization of DL-lactate was examined in slants prepared with the medium containing DL-lactic acid 60% (w/v) -2.5ml, NaCl - 5.0g, K_2HPO_4 - 1.0g, $NH_4H_2PO_4$ - 1.0g, $MgSO_4.7H_2O$ - 0.2g, Agar - 15.0g, Bromothymol blue 0.2% - 0.4mL, distilled water - 1L with pH.6.8. The slants were heavily inoculated with the test organisms and were incubated at 28^oC. Formation of deep blue color was an indication of DL-lactate utilization (Janda *et al*., 1996).

5.2.8.4.7 ***Acid production from Sugars***

Hugh and Leifsons basal medium was used for this purpose, which contained, Peptone - 2.0g, NaCl - 5.0g, K_2HPO_4 - 0.3g, Phenol red - 30ml, distilled water - 1L with pH.7.3 ± 2.0.The carbohydrates such as sucrose, D-cellobiose and salicine were added to a final concentration of 0.1% (w/v). Acid production was readily observed by incorporating into the medium an appropriate pH indicator (e.g. phenol red). The tubes were inoculated with a needle and incubated at 28±0.5^oC for 3days and the

results recorded. Production of acid induced a change in the phenol red indicator, from pink to yellow under acidic conditions.

5.2.9 Determination of Digestibility of yeast feed supplement in Koi carp

The digestion of yeast supplement in feed by koi carp was determined by the analysis of the faecal matter.

5.2.9.1 Collection of faecal matter

Digestibility was assessed by collecting fish faeces from the tanks (Belal, 2005). Fishes were fed the experimental diet for an hour and, all uneaten feed pellets removed subsequently and the tanks thoroughly cleaned to remove faeces and bacterial slime. Faecal matter was collected by manual siphoning using silicone tube or pipetting from the bottom of the tanks depending on convenience. Collected faecal material was centrifuged at 10000 x g suspended in distilled water and observed under light, dark field and phase contrast microscopes.

5.2.10 Statistical analysis

All data were evaluated to determine the effect of the yeast supplementation on growth performance, haematological parameters and survival after challenge by One-way Analysis of Variance (ANOVA) with post-hoc multiple comparison analysis performed using Tukey's HSD using SPSS 15.0 package for Windows at a significance level of $p<0.05$ (Appendix 2). Data are presented as mean± standard deviation.

5.3 Results

5.3.1 Growth

The initial weight of the fish was 1± 0.2g in all groups. The fishes fed with yeast supplemented diet weighed significantly higher ($p< 0.05$) after

13 weeks of feeding (Fig.5). The results showed that the growth performance of fish fed with diets containing different levels of dietary yeast varied. Growth performance and feed utilization increased significantly ($p< 0.05$) in the batches of fishes administered with both live and inactivated yeast supplementation as evaluated by weight gain, SGR, FE, FCR, PI, PER (Table.1 & 2). The higher growth observed was when live yeast was administered at 10^6cfu g^{-1} (10.47 ± 1.03g) ($p < 0.05$) followed by inactivated yeast at 10^8cfu g^{-1} (7.96 ± 0.85g) ($p< 0.05$). However, low growth was observed when fed on live yeast at an elevated concentration of 10^9cfu g^{-1} (4.07±0.33g) ($p> 0.05$) compared with the batch of fishes fed on the diet not supplemented with yeast (6.22±0.79g). Moreover, when fishes were fed with diets containing live yeast at the concentration of 10^6cfu g^{-1} and inactivated yeast at 10^8cfu g^{-1} they consumed more feed than the batches of fishes with other treatments with lowest FCR, such as 2.27±1.50 and 2.56±0.19 respectively. Meanwhile, fishes fed with live and inactivate yeast at 10^9cfu g-^{1} consumed less feed giving a higher FCR (3.80±0.24 and 3.34±0.47). On the other hand, yeast supplementation in general improved nutrient utilization and the fishes fed on live yeast at 10^6cfu g^{-1} (1.30±0.28) and 10^8cfu g^{-1} (1.26±0.43) followed by inactivated yeast at 10^8cfu g^{-1} (1.12±0.08) had the highest PER; the lowest PER was observed with fishes fed on live yeast at 10^9cfu g^{-1} (0.66±0.04). Fishes fed with live yeast at 10^6cfu g^{-1} had the highest weight gain, SGR, FE, PI and PER.

5.3.2 Physiological parameters

Physiological parameters (Haematological and biochemical) are shown in Table 3 & 4.

5.3.2.1 Haematological indices

Haemoglobin content was significantly ($p \leq 0.05$) higher as compared to control only in the batches of fishes fed on live yeast at 10^6cfu g^{-1} (7.83 ± 0.20g dL^{-1}) and followed by inactivated yeast at 10^9cfu g^{-1} (7.5±0.20 g dL^{-1}). There was no significant difference between the other test groups. WBC count in different treatment groups did not show any significant ($p> 0.05$) difference; however, it was significantly higher ($p< 0.05$) in the control group (666 ± 230.9×10^3 μL^{-1}). RBC count did not show any significant ($p> 0.05$) difference in between the groups of fishes fed with different doses/concentration of yeast and the control group. In the same, PCV was not significantly ($p> 0.05$) different between the groups of fishes fed on different doses/concentration of yeast and the control. Fishes fed with diets containing live yeast 10^6cfu g^{-1} exhibited higher Hb, RBC, and PCV ($p<0.05$).

5.3.2.2 Biochemical indices

A significantly higher blood glucose level was found in the group of fishes fed with live yeast at 10^6cfu g^{-1} (82 ± 16.64mg dL^{-1}) which got reduced significantly in the group fed with live yeast at 10^9cfu g^{-1} (38.3 ± 1.53 mg dL^{-1}) compared to that of the control (48.3 ± 6.66 mg dL^{-1}). Serum protein level registered slight increase in the batches of fishes fed on live yeast at 10^6cfu g^{-1} (2.83 ± 0.29g dL^{-1}) and exhibited significant reduction in the batches fed with live yeast at 10^9cfu g^{-1} (1 ± 0.17 g dL^{-1}) compared to the one recorded in the control (2.63 ± 0.38 g dL^{-1}). Albumin levels were significantly influenced by *Candida* MCCF 101 supplement, as the lowest level was observed in the fishes fed on live yeast at 10^9cfu g^{-1} (0.83 ± 0.06g dL^{-1}) and the highest in the fishes fed with inactivated yeast at 10^8cfu g^{-1}

(2.51±0.14 g dL^{-1}). Globulin levels did not significantly (P> 0.05) differ between the treatments groups with live and inactivated yeast concentration at 10^6cfu g^{-1} to10^9cfu g^{-1} compared with that of the control group. Yeast supplementation increased glucose, globulin and protein values higher in live yeast fed groups at 10^6cfu g^{-1}.

5.3.3 Challenge study

5.3.3.1 LD_{50} of *Aeromonas* sp. MCCB 113

The virulence of *Aeromonas* MCCB 113 was assessed *in vivo* from the LD_{50} value. Koi carp (*Cyprinus carpio haematopterus*) was used as the test model and LD_{50} of the *Aeromonas* sp. MCCB 113 was found to be $10^{7.1}$cfu mL^{-1} as summarized in Table.5.

5.3.3.2 Challenge Experiment

On challenging fishes with *Aeromonas* sp. MCCB 113, mortality was observed within the first 10 days. There was no mortality due to handling stress as all fishes survived for 12 hrs post challenge. The highest survival was shown in the group of fishes fed on live yeast at 10^6cfu g^{-1} (91.67%) followed in the group fed on inactivated yeast at 10^7cfu g^{-1} (83.3%), whereas, the lowest survival was obtained in the group of fishes fed with live yeast at 10^8cfu g^{-1} (25%) compared with that of the control (58.3%) (Fig.6).

External signs such as reddening of the site of injection appeared in both experimental and control groups as early as one hour post injection. However, further signs of haemorrhagic pockets, loss of scales, necrotic lesions and swelling and the formation of open wounds were observed only in fishes which died due to the infection (Fig.7 a-d).

5.3.3.3 Identification of the organism re-isolated

The organisms re-isolated from the liver of moribund fishes of different groups were phenotypically characterized and found matching with the characteristics of *Aeromonas* sp. MCCB 113. The isolates, which are Gram-negative, rods, motile, oxidase positive, glucose fermenting, resistant to O/129 are designated to be *Aeromonas* (Erova *et al*., 2007). The phenotypic characterization of the isolated organism from different groups of infected fishes and their identification of *Aeromonas* sp. MCCB 113 has been summarized in Table.6.

5.3.4 Microscopic examination of faecal matter

Microscopic observation of faecal pellets of koi carp was conducted with light, dark field and phase contrast microscopes. The negative control used was the feed without yeast supplementation whereas the positive control was the feed supplemented with live and inactivated yeasts. The test samples were the faecal matter collected from the experimental tanks. The faecal matter on microscopic observation did not show the presence of any intact yeast cell in different samples taken. Meanwhile in the positive control intact yeast cells could be seen in both live and inactivated yeast coated feeds (Fig. 8 a-c, 9 a-c, 10 a-c, 11 a-c, 12 a-c). This observation strongly suggested that yeast cells could be digested in the gut of koi carp.

5.4 Discussion

Yeast-based products have been used in aqua feeds for increased growth, feed intake and disease resistance (Ortuno *et al*., 2002; Li and Gatlin III, 2003; 2004; 2005). Yeast single cell protein sources provide superior and better nutritional value in fish diets than other SCP sources.

This may be due to its acceptability, palatability and digestibility compared to the other SCPs (Bob-Manuel and Alfred-Ockiya, 2011). Marine yeast *Candida* MCCF 101 supplemented feed was evidently beneficial to koi carp (*Cyprinus carpio haematopterus*) indicated by the decreased mortality on challenge and increased growth rate. Yeast supplementation of koi carp diets might have made the diets more palatable, increasing feed intake and subsequent weight gain.

In the present study, the supplementation of live yeast, *Candida* MCCF 101 improved growth and feed utilization. The result of this study clearly showed that live yeast at 10^6cfu g^{-1} followed by inactivated yeast at 10^8cfu g^{-1} as dietary feed supplements enhanced the growth of koi carp, whereas live and inactivated yeast concentration of 10^9cfu g^{-1} depressed the growth, even below to that of fishes fed with the control diet. Tovar *et al*. (2002); Lara-Flores *et al*. (2003); Wache *et al*. (2006) and Abdel-Tawwab *et al*. (2008) found that the addition of live yeast improved diet and protein digestibility which might explain better growth and feed efficiency obtained with yeast supplements. The improved fish growth and feed utilization may possibly be due to the improved nutrient supplementation and digestibility. These results agree with those obtained with Catla (Mohanty *et al*., 1996), Mrigal (Swain *et al*., 1996), Hybrid striped bass (Li and GatlinIII, 2003; 2004; 2005) and Japanese flounder (Taoka *et al*., 2006). Similar results were obtained when *S. cerevisiae* was added to fish diet for Israeli carp (Noh *et al*., 1994) and Nile tilapia (Lara- Flores *et al*., 2003). Present study strongly suggests that the higher dietary levels of yeast can even negatively influence the physiological status and growth of koi carp. At higher concentration, the yeast may be enhancing the innate

immune system acting as a chronic stressor resulting in high cortisol levels inhibiting growth.

Feed utilization was highest in carp fed with live yeast (10^6cfu g^{-1}) supplemented diet suggesting that the nutrients were more efficiently utilized for growth and energy. These results suggest that yeast supplementation plays a significant role in enhancing feed intake with subsequent enhancement of growth rate. The better feed intake might be due to increased appetite resulting in improved growth. Abdel-Tawwab *et al*. (2010) stated that better feed utilization with yeast supplementation might have been because of its possible role in enhancing feed intake and digestibility resulting in higher growth. On the other hand, changes in protein and lipid content in fish body could be linked with changes in their synthesis, rate of deposition and differential growth rate (Smith, 1981; Fauconneau, 1984; Soivio *et al*., 1989; Abdel-Tawwab *et al*., 2006).

Lowest feed efficiency was observed when live yeast was fed at the higher rate at 10^9cfu g^{-1} (0.26±0.02) compared with the control fish not fed with yeast (0.39±0.04). The study suggested that increasing the level of yeast resulted in reduced palatability and reduced feed intake and reduced growth. Similar to the results recorded here, Rumsey *et al*. (1991b) demonstrated decreased feed intake in rainbow trout with higher levels of yeast in the diet; diets with greater than 50% yeast were unpalatable to rainbow trout.

Protein intake was not significantly different between the groups fed with live yeast and inactivated yeast, however, the highest protein intake was observed for the fishes fed on live yeast at the rate 10^6cfu g^{-1}. Protein

intake was proportionally utilized for growth and as energy sources, as lipids and carbohydrates were adequately utilized to meet the energy requirements. Improved growth and feed conversion efficiency are linked with increasing dietary protein levels as observed by Lazo *et al*. (1998). PER was significantly higher in fishes fed with live yeast at 10^6cfu g^{-1} (1.30±0.28) and inactivated yeast at 10^8cfu g^{-1} (1.12±0.08) than that of the control fishes (1.0±0.10). The high PER observed may be due to the amino acid profile which stimulated growth. Low PER was observed in fishes fed with both live and inactivated yeast at a concentration of 10^9cfu g^{-1} (0.66±0.04 and 0.85±0.11). Yeast is a source of nucleic acids and non-starch polysaccharides, including β-1, 3 glucan, which in high concentrations may play a role as antinutritional factors. At high concentrations, such compounds are known to hamper digestion and/or absorption.

Biochemical analyses often provide vital information for health assessment and management of cultured fish (Pincus, 1996; Cnaani *et al*., 2004; Rehulka *et al*., 2004). In the present study, fishes fed with live yeast at 10^6cfu g^{-1} exhibited higher RBCs, Hb, PCV, glucose, albumin, globulin and protein values. However, on feeding with increased concentration of yeast above 10^6cfu g^{-1} those parameters were found declining. These results suggested that on feeding fishes with yeast at moderate level there is an overall improvement of health of the fishes. Serum components varied widely between the experiments. Dietary incorporation of inactivated yeast appeared to have no significant effect on haematology especially in glucose, albumin, globulin and protein levels. The measurement of albumin, globulin, and total protein in serum or plasma is of considerable

diagnostic value in fish, as it relates to general nutritional status as well as to the integrity of the vascular system and liver function.

Aeromonas, one of the major bacterial fish pathogen, is known to cause a variety of diseases such as haemorrhagic septicaemia, infectious dropsy, tropical ulcerative disease and fin rot leading to heavy mortality in culture farms (Kumar and Dey, 1988; Karunasagar *et al*., 1997). Present study demonstrates that supplementation of yeast *Candia* MCCF 101 as live and inactivated form has a positive influence on the survival of Koi carp by resisting to *Aeromonas* sp. MCCB 113 infection. In this study the highest percentage survival was observed in live yeast fed at 10^6cfu g^{-1} (91.67%) followed by inactivated yeast fed at 10^7cfu g^{-1} (83.33%), which were higher than that of the other groups. The presence of yeast in the diet may enhance the innate immune response of fish (Ortuno *et al*., 2002). Yeast supplementation may also have improved survival by providing nutritional benefits, enhancing fish health by immunomodulation, or some combination of these actions in conjunction with palatability. The survivability in the group of fishes fed on live yeast of 10^8cfu g^{-1} and 10^9cfu g^{-1} was lower than that of the control group. Over stimulation of the immune system however, may be acting as the chronic stressor resulting in constant production of cortisol, even in the unstressed fish, causing immune exhaustion. Chronic immunostimulation may also inhibit lean muscle growth (Johansen *et al*., 2006).

It is also possible that the increased growth and decreased mortality of fishes fed with yeast was due to yeast immunomodulatory effects. Tovar-Ramirez *et al.* (2004) observed similar mortality and growth patterns in European sea bass-fed diets containing a different yeast species,

Debaryomyces hansenii. Yeast in general has been shown to improve immunological function in fish (Siwicki *et al*., 1994; Nakano *et al.,* 1995; 1999; Ortuno *et al*., 2002; Li and Gatlin III, 2004; 2005)

Microscopic examination confirmed digestion of yeast cell in Koi carp. This indicated that the yeast cells were broken or damaged up on passing through the gut of the fish. A long term digestibility trial is required for better understanding on the digestibility and utilization of yeast based feeds. The undigested food in the faecal matter is a potential hazard to water quality and will likely raise yeast levels in receiving water bodies. Any feed with low digestibility negatively affects economy of the culture as well.

The present study indicated that live marine yeast *Candida* MCCF 101 positively enhanced growth performance and feed utilization of koi carp as well as its resistance to *Aeromonas* sp. MCCB 113 infection. The optimum levels of both live and inactive yeast were 10^6cfu g^{-1} and 10^8cfu g^{-1} respectively. It has been concluded that the marine yeast isolate, *Candida* MCCF 101 provides better nutritional and dietary values, positively enhance growth performance, immunity and survival. All these qualities lead us to recommend it as a feed supplement in aquaculture especially in Koi carp.

Table.1 Growth performance and feed efficiency of Koi carp fed with live yeast

			Live yeast (cfu/mL^{-1})			
	Items	**Control**	**10^6**	**10^7**	**10^8**	**10^9**
1	Initial wt (g)	1.03±0.06^{a}	1.08±0.04^{a}	1.02±0.05^{a}	1.03±0.23^{a}	0.94±0.03^{a}
2	Final wt(g)	6.22±0.79^{bc}	10.47±1.03^{a}	7.57±0.92^{b}	7.38±0.92^{b}	4.07±0.33^{c}
3	Weight gain(g)	5.19±0.81^{bc}	9.39±1.05^{a}	6.55±0.92^{b}	6.35±1.15^{b}	3.13±0.32^{c}
4	Specific growth rate(g)	1.97±0.17^{bc}	2.49±0.13^{a}	2.20±0.15^{b}	1.98±0.36^{bc}	1.61±0.09^{c}
5	Feed efficiency	0.39±0.04^{ab}	0.46±0.10^{a}	0.35±0.02^{ab}	0.45±0.15^{ab}	0.26±0.02^{b}
6	FCR	2.85±0.30^{a}	2.27±1.50^{a}	2.91±0.67^{a}	2.42±0.90^{a}	3.80±0.24^{a}
7	Protein intake (%)	5.16±0.50^{bc}	7.33±0.78^{a}	6.59±0.49^{ab}	5.23±0.91^{bc}	4.77±0.45^{c}
8	PER	1.0±0.10^{ab}	1.30±0.28^{a}	1.00±0.21^{ab}	1.26±0.43^{ab}	0.66±0.04^{b}

Mean values having the same superscript in the same row are not significantly different at P≤0.05.

Table.2 Growth performance and feed efficiency of Koi carp fed with inactivated yeast

			Inactive yeast (cfu/mL^{-1})			
	Items	**Control**	**10^6**	**10^7**	**10^8**	**10^9**
1	Initial wt (g)	1.03±0.06^{a}	1.12±.34^{a}	1.04±0.16^{a}	1.13±0.01^{a}	1.1±0.14^{a}
2	Final wt(g)	6.22±0.79^{a}	6.33±1.60^{a}	7.54±0.34^{a}	7.96±0.85^{a}	6.2±0.76^{a}
3	Weight gain(g)	5.19±0.81^{ab}	5.38±1.37^{b}	6.49±0.20^{b}	6.84±0.85^{a}	5.1±0.66^{ab}
4	Specific growth rate(g)	1.97±0.17^{ab}	1.91±0.42^{b}	2.18±0.12^{a}	2.14±0.11^{a}	1.90±0.9^{ab}
5	Feed efficiency	0.39±0.04^{a}	0.36±0.04^{ab}	0.36±0.01^{a}	0.39±0.03^{a}	0.30±0.0^{b}
6	FCR	2.85±0.30^{a}	2.76±0.27^{a}	2.80±0.04^{a}	2.56±0.19^{a}	3.34±0.47^{a}
7	Protein intake (%)	5.16±0.50^{a}	5.07±0.96^{a}	6.32±0.24^{a}	6.08±0.32^{a}	6.09±1.19^{a}
8	PER	1.0±0.10^{ab}	1.05±0.11^{ab}	1.03±0.01^{ab}	1.12±0.08^{a}	0.85±0.11^{b}

Mean values having the same superscript in the same row are not significantly different at P≤0.05.

Table.3. Haematological and biochemical parameters of Koi carp fed with live yeast

		Live yeast (cfu mL^{-1})			
Items	**Control**	**10^6**	**10^7**	**10^8**	**10^9**
HB (g/dl)	4.8 ± 0.61^{b}	7.83 ± 0.20^{a}	5.57 ± 1.32^{ab}	4.93 ± 1.45^{ab}	6.9 ± 2.19^{ab}
RBC (10^6/µL)	0.72 ± 0.18^{a}	1.14 ± 0.20^{a}	0.75 ± 0.36^{a}	0.68 ± 0.15^{a}	1.2 ± 0.16^{a}
WBC (10^3/µL)	666 ± 230.9^{a}	333.35 ± 7.7^{a}	416.67 ± 160.7^{a}	383.33 ± 125.8^{a}	283.3 ± 125.8^{a}
PCV (%)	14 ± 2.80^{a}	25.76 ± 2.86^{a}	25.15 ± 8.53^{a}	14.63 ± 3.70^{a}	20.2 ± 5.16^{a}
Glucose (mg/dL)	48.3 ± 6.66^{bc}	82 ± 16.64^{a}	65.3 ± 3.51^{ab}	46 ± 1.73^{bc}	38.3 ± 1.53^{c}
Albumin (g/dL)	1.84 ± 0.29^{a}	1.83 ± 0.29^{a}	1.33 ± 0.58^{ab}	1.35 ± 0.14^{ab}	0.83 ± 0.06^{b}
Globulin (g/dL)	0.8 ± 0.60^{a}	1 ± 0.50^{a}	0.83 ± 0.06^{a}	0.52 ± 0.17^{a}	0.17 ± 0.11^{a}

Mean values having the same superscript in the same row are not significantly different at P≤0.05.

Table.4 Haematological and biochemical parameters of Koi carp fed with inactivated yeast

		Inactive yeast (cfu mL^{-1})			
Items	**Control**	**10^6**	**10^7**	**10^8**	**10^9**
HB (g/dl)	4.8 ± 0.61^{a}	4.05 ± 2.34^{a}	4.4 ± 1.75^{a}	5.9 ± 1.49^{a}	7.5 ± 0.20^{a}
RBC (10^6/µL)	0.72 ± 0.18^{a}	0.8 ± 0.46^{a}	0.79 ± 0.18^{a}	0.84 ± 0.27^{a}	1.14 ± 0.20^{a}
WBC (10^3/µL)	666 ± 230.9^{a}	325 ± 256.6^{ab}	200 ± 100^{b}	250 ± 132.3^{b}	166.6 ± 57.74^{b}
PCV (%)	14 ± 2.80^{a}	12.2 ± 7.16^{a}	14.3 ± 6.88^{a}	19 ± 7.67^{a}	24.4 ± 2.86^{a}
Glucose (mg/dL)	48.3 ± 6.66^{b}	63.7 ± 1.53^{a}	70 ± 2.00^{a}	66.3 ± 3.51^{a}	40.3 ± 3.21^{b}
Albumin (g/dL)	1.84 ± 0.29^{ab}	1.4 ± 0.36^{b}	1.42 ± 0.32^{b}	2.51 ± 0.14^{a}	1.55 ± 0.35^{b}
Globulin (g/dL)	0.8 ± 0.60^{a}	0.77 ± 0.31^{a}	0.88 ± 0.63^{a}	0.19 ± 0.08^{a}	0.81 ± 0.44^{a}
Protein (g/dL)	2.63 ± 0.38^{a}	2.17 ± 0.57^{a}	2.3 ± 0.70^{a}	2.7 ± 0.17^{a}	2.37 ± 0.76^{a}

Mean values having the same superscript in the same row are not significantly different at P≤0.05

Table.5 Test of pathogenicity of *Aeromonas* sp. MCCB 113 in Koi carp

Bacterial Dose	Infected test units	Cumulative infected (A)	Cumulative non infected (B)	Ratio A/A+B	% of infected (%)
1×10^{8}	8/8	13	0	13/13	100
1×10^{7}	4/8	5	4	5/9	55.5
1×10^{6}	1/8	1	11	1/12	8.33
1×10^{5}	0/8	0	11	0/11	0
1×10^{4}	0/8	0	11	0/11	0
Control	0/8	0	11	0/11	0

$LD_{50} = 10^{7.1}$ CFU/mL

Table.6 Phenotypic characterization of the bacterial isolates from infected Koi carp fed with different dose of yeast, *Candida* MCCF 101

Phenotypic charecterestic	*A.Caviae*	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9
Gram's stain	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+
MOF	F	F	F	F	F	F	F	F	F	F
Kovac's oxidase	+	+	+	+	+	+	+	+	+	+
O/129 sensitivity	-	-	-	-	-	-	-	-	-	-
Utilization of:										
1. DL- lactate	-	-	-	-	-	-	-	-	-	-
Acid production from:										
1. Sucrose	+	+	+	+	+	+	+	+	+	+
2. D-Cellobiose	+	+	+	+	+	+	+	+	+	+
3. Salicine	+	+	+	+	+	+	+	+	+	+

(a)

(b)

(c)

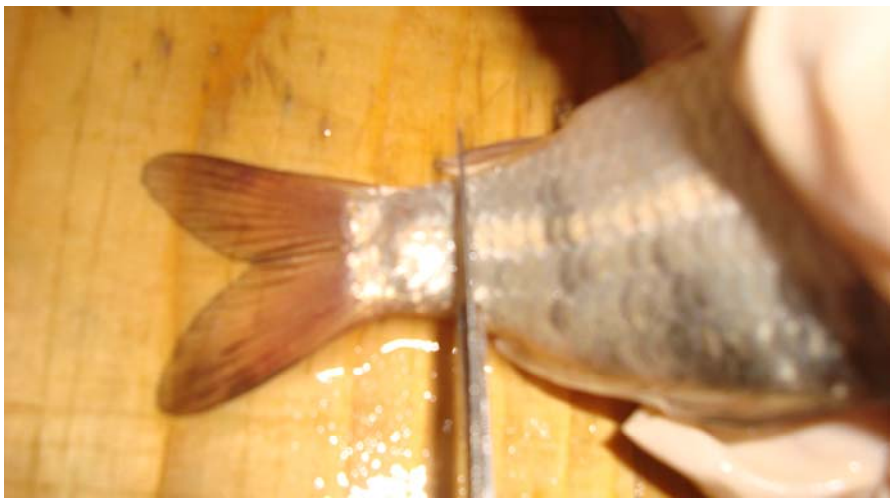
Fig.1(a-c) Over view of feeding experimental setup

(a)

(b)

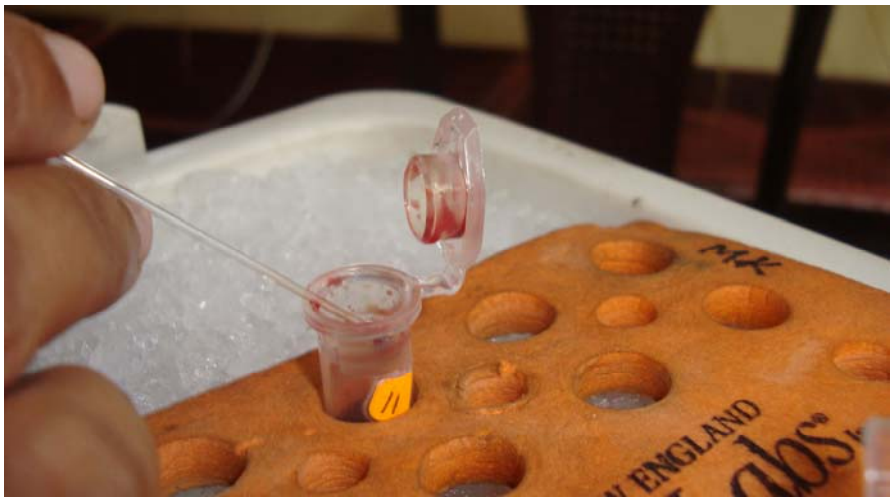
Fig.2 (a-b) Fish anaesthetized with clove oil

(a)



(b)

(c)



(d)

Fig.3 (a-d) Blood collection by tail ablation

(a)

(b)

(c)

(d)

Fig.4 (a-d) Lethal dose (LD_{50}) determination by intraperitoneal (IP) injection and the experimental set up

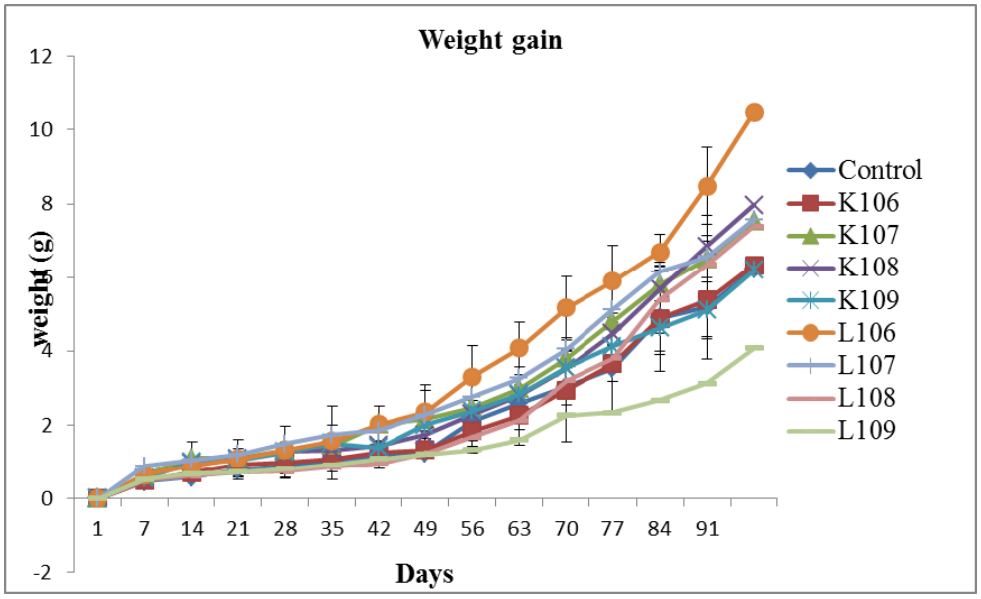


Fig.5 Yeast supplementation as evaluated by weight gain

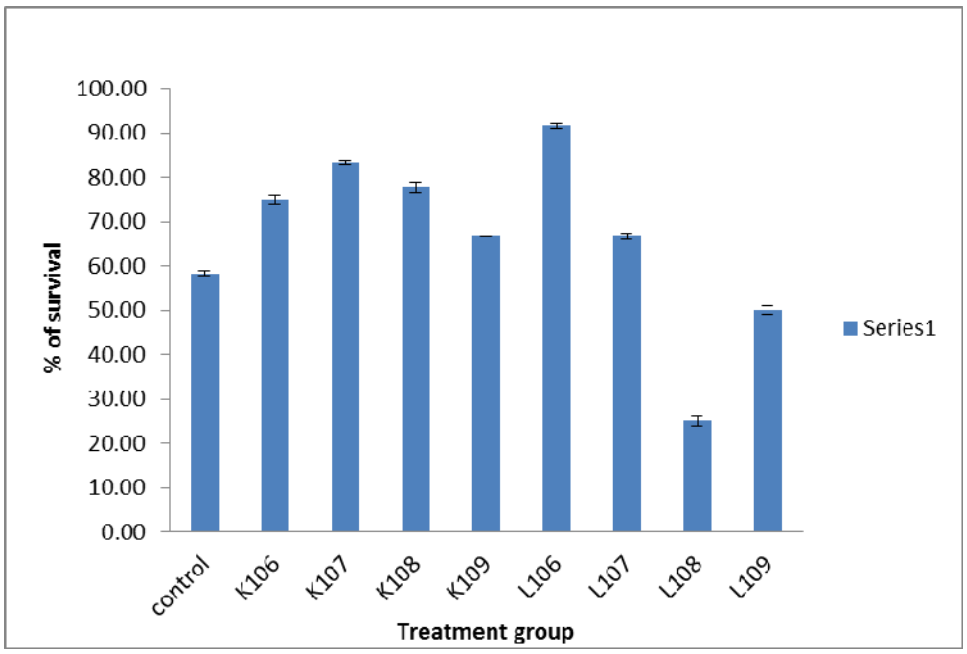
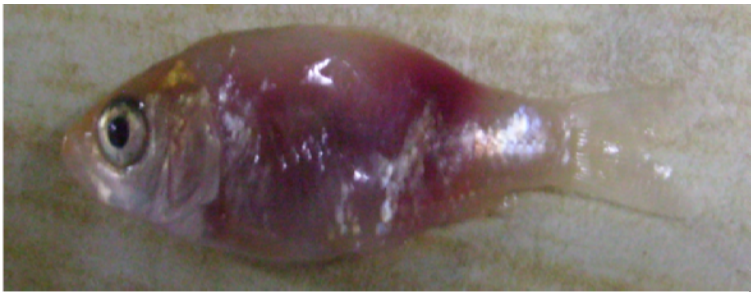


Fig.6 Percentage survival of Koi carp after challenge with *Aeromonas* sp. MCCB 113

(a) Swollen abdomen

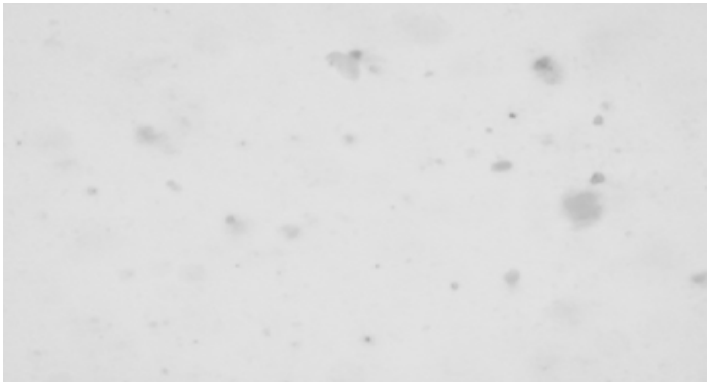


(b) Skin lesion

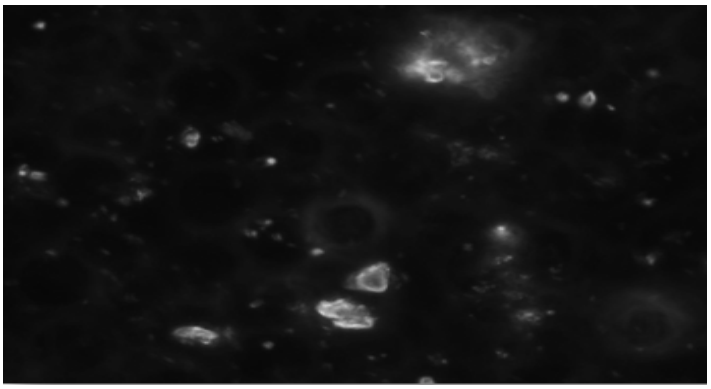
(c) Haemorrhages

(d). Scale loss

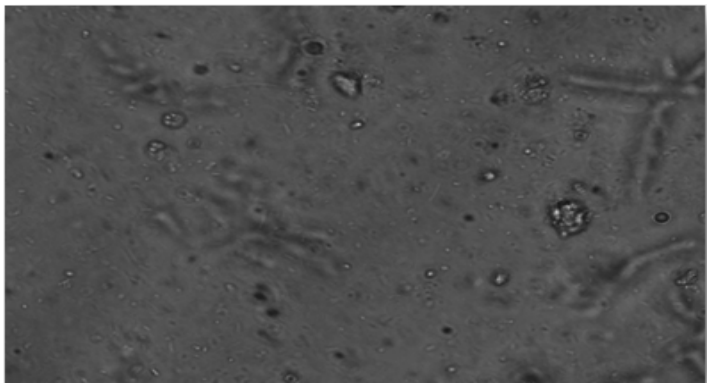
Fig.7 (a- d) Clinical signs of fish dead of *Aeromonas* sp. MCCB 113 infection



(a). Light microscopy

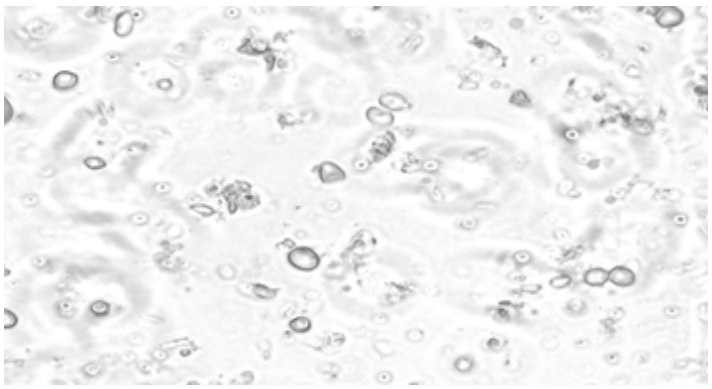


(b). Dark field microscopy

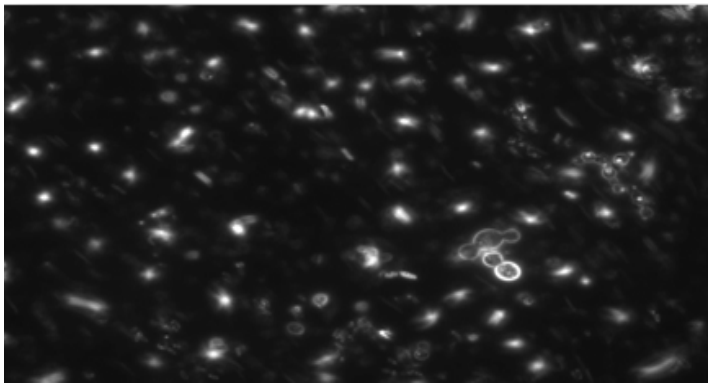


(c). Phase contrast microscopy

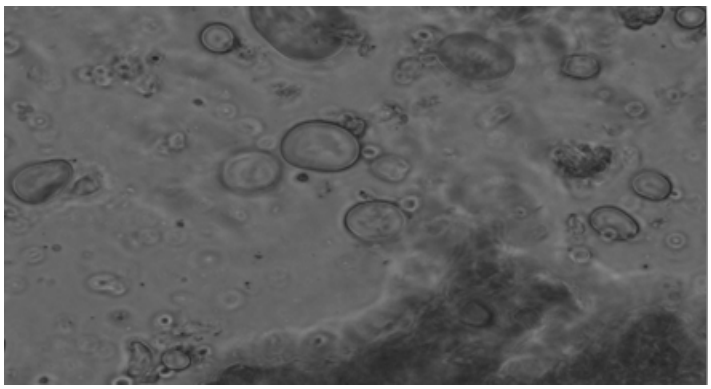
Fig.8 (a-c) Negative control (feed only)



(a) Light microscopy

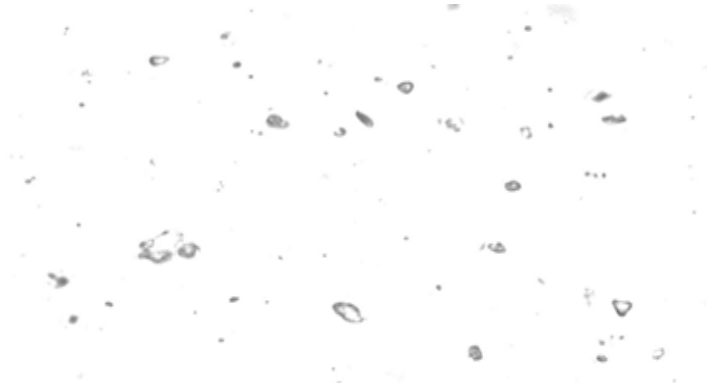


(b) Dark field microscopy

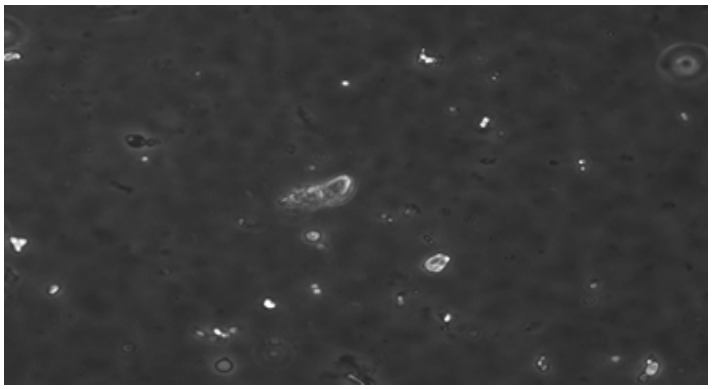


(c) Phase contrast microscopy

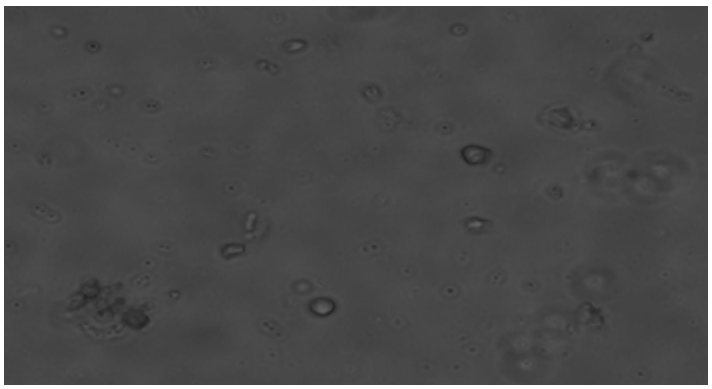
Fig.9 (a-c) Positive controls (feed with live yeast)



(a) Light microscopy

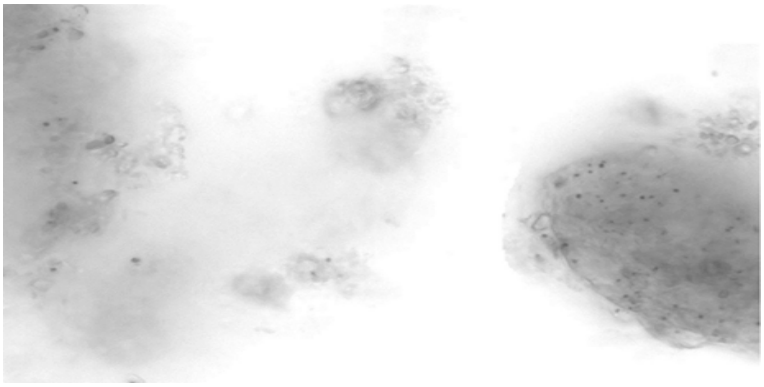


(b) Dark field microscopy

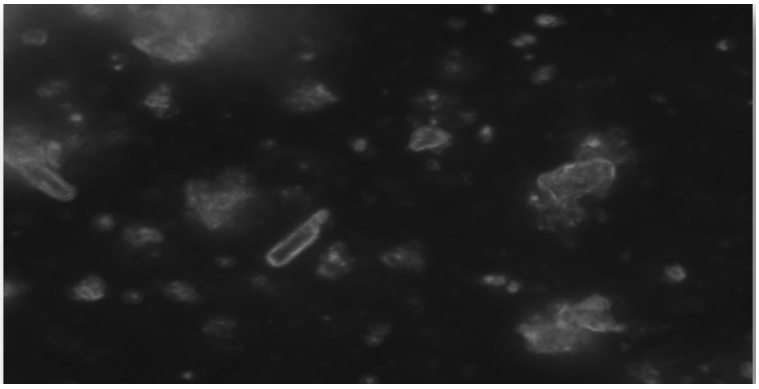


(c) Phase contrast microscopy

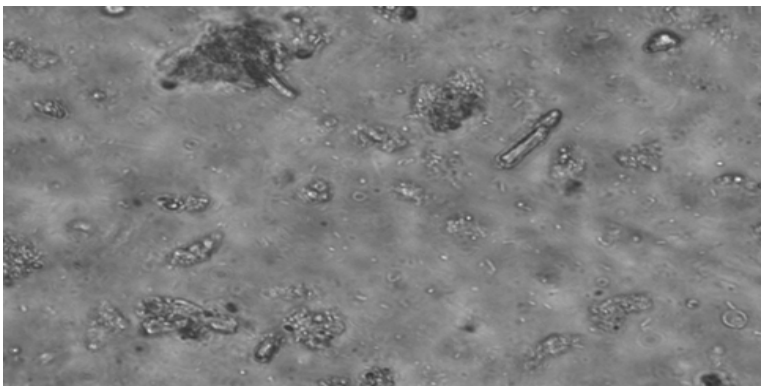
Fig.10 (a- c) Positive controls (feed with inactivated yeast)



(a) Light microscopy

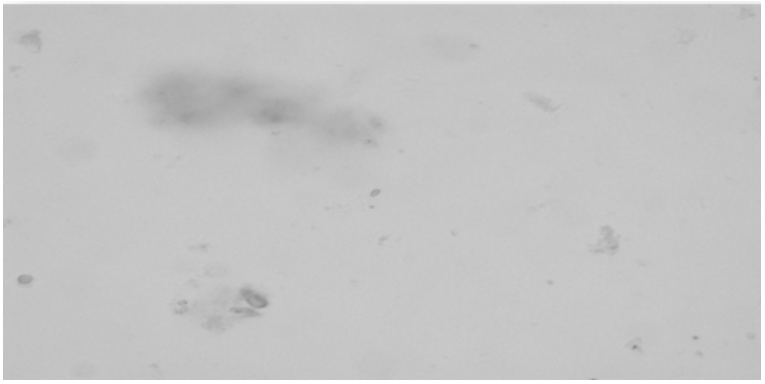


(b) Dark field microscopy

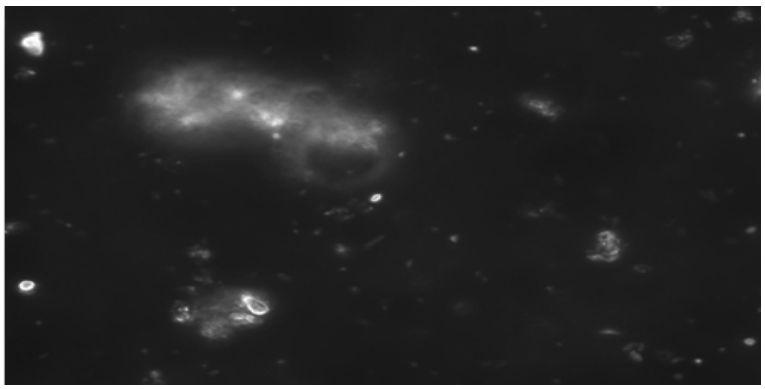


(c) Phase contrast microscopy

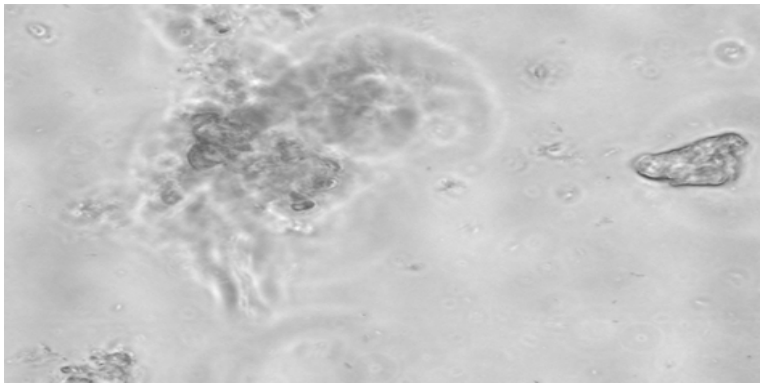
Fig.11 (a- c) Faecal matter of fish fed with live yeast



(a) Light microscopy



(b) Dark field microscopy



(c) Phase contrast microscopy

Fig.12 (a- c) Faecal matter of fish fed with inactivated yeast

Chapter 6

CONCLUSIONS AND SCOPE FOR FUTURE RESEARCH

Aquaculture feed market has become ever more sophisticated in the recent years with the emergence of new products with improved quality. Single cell protein (SCP) appears to be an attractive entrant into this growing list. Adequate nutrition has long been acknowledged as crucial for maintaining animal health and disease resistance. Advancements in fish nutrition have progressed considerably with regard to growth performance. However, much concern has arisen over the quality of fish flesh and health in aquaculture. Dietary supplements fed within safety limits will result in beneficial effects for the animals. A variety of feed supplements have been investigated with respect to their effects on health and quality of cultured fish. Single cell proteins, including yeast and bacteria, have been viewed as promising substitutes for fishmeal in fish diets. Various species of yeast and different yeast-containing products have been fed to fish for an assortment of reasons. Yeast protein is known for its easily digestible nutritious value and has been widely used as animal and aquaculture feed supplement. Some yeasts used as sources of SCP are *Pichia*, *Candida*, *Saccharomyces*, *Kluyveromyces, Torulopsis*, *Hansenula, Koloechera* etc. Utilization of whole cell yeast instead of the cell wall component β-l, 3 glucan warrants much importance due to the nutritional quality of the yeast biomass (Tacon, 1994; Ravindra, 2000; Li and Gatlin, 2003; Zhenming *et al*., 2006; Sajeevan *et al*., 2009a). The present study shows that the marine yeasts provide better nutritional and dietary values, serve as good

source of immunostimulants and increase biomass production indicating their potential application as feed supplement in aquaculture.

Significance of the present study

Over the years marine yeasts have been gaining increased attention in animal feed industry due to their nutritional value and immune boosting property. Marine yeasts are regarded as a safe feed material easily amenable to biotechnological processes. Marine yeasts have high protein and carbohydrate content, high levels of saturated fats and a balanced amino acid composition. However, there is paucity of information on marine yeasts as feed supplements and no feed formulation has been found either in literature or in market supplemented with them. Aquaculture field still lacks a feed composed of single cell protein (SCP) from marine yeast with high content of protein and other nutrients. Sajeevan *et al*. (2006; 2009a) observed that marine yeast *Candida* MCCF 101 had immunostimulatory properties in cultured shrimps. In order to bring out its comprehensive benefits for aquaculture, the present study was undertaken for assessing its nutritional value, developing a bioprocess technology for its commercial production and evaluating its impact on growth and disease resistance in koi carp.

Objectives of the investigation

1) Identification of the marine yeast isolate *Candida* MCCF 101 and its proximate composition

2) Optimization of physical parameters such as pH, temperature and NaCl content in malt extract broth using Response Surface Methodology (RSM) and generation of yeast biomass in laboratory and pilot scale fermentors.

3) Optimization and scale-up of yeast biomass production in mineral based medium and comparison with that in a commercial fermentation medium.

4) The marine isolate *Candida* MCCF101 as dietary feed supplement to enhance growth, immunocompetency and survival of Koi carp (*Cyprinus carpio haematopterus*) challenged with *Aeromonas* sp. MCCB 113.

The important findings of the study are summarized as follows:

- Morphological observation showed that *Candida* MCCF101 produced elliptical or club shaped cells of 5µm diameter. Due to the large size (5µm) the cells settle leaving a clear supernatant when kept undisturbed. Moreover, it can be sedimented by centrifugation at 7500 rpm.

- Based on sequence analysis of internal transcribed spacers (ITS) the yeast isolate showed a similarity of 98% to *Candida* sp.S27 and 97.8 % to *Candida aquae-textoris* ATCC 20145. The nucleotide sequence has been deposited in GenBank and assigned the Accession No: FJ652052.

- Proximate composition of *Candida* MCCF101 revealed protein - 30 ±1.63%, carbohydrate - 36.25 ±1.25%, lipid - 1.52 ± 0.04%, nucleic acid - 12.05 ± 0.05%, dietary fibre - 10.05 ± 0.50%, ash - 6.67 ± 0.94% and wet yeast biomass contained approximately77 ± 0.50% moisture.

- Total free amino acid (FAA) content was estimated as 16.16% of the total amino acids of yeast.

- *Candida* MCCF 101 contained essential and non-essential amino acids such as aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine when estimated by HPLC. Tryptophan which was estimated separately by spectrophotometry accounted for 6.5% of total protein. Among the amino acids glutamic acid and leucine were of highest concentration.

- Fatty acid profile exhibited long chain fatty acids (up to 18-carbon atoms) along with polyunsaturated fatty acids (PUFA). Fatty acids recorded were lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid, margaroic acid, margaric acid, linoleic acid and stearic acid. Among them, linoleic acid, stearic acid and palmitic acid were dominant.

- Optimization of inoculum was done through analysis of variance (ANOVA), which showed that there were no significant differences (P value=0.96033) in inoculum size from 0.025 ($1.39x10^5$cfu mL^{-1}) to 0.2 ($11.14x10^5$cfu mL^{-1}) absorbance at Abs_{540nm}. Hence the cell suspension having absorbance 0.1 at Abs_{540nm} ($5.57x10^5$cfu mL^{-1}) was chosen as inoculum for further studies.

- The physical parameters such as salinity, pH and incubation temperature were screened by conventional one-factor at a time analysis and further optimized statistically by response surface methodology (RSM) using the software Design-Expert (version 6.0.9, Stat-Ease, Minneapolis, MN, USA).

- The most popular response surface methodology design is full factorial central composite design (CCD), and has been used to predict the maximum biomass at different combinations of sodium chloride concentration, pH, and temperature. A total of 20 different experiments (2^3 full factorial points, 6 axial points and 6 center points) were suggested by the software for optimization of culture conditions.
- Statistical testing of the method was done by standard analysis of variance (ANOVA). The analysis of variance of the quadratic regression model demonstrated that the model was highly significant (P>0.0001) for biomass production. The model F-value 25.07 for biomass also implied that the model was significant.
- Goodness of fit of the model was checked by coefficient of determination (R^2). R^2 was 0.9576 in the case of biomass production. As per the model, the sample variation of 95.76% was attributed to the independent variables and the model did not explain only 4.24% of the total variation. A higher value of the correlation coefficient (r = 0.9786) indicated that an excellent correlation existed between experimental and predicted values.
- The value of "Pred R^2" of 0.7283 was in reasonable agreement with the "Adj R^2" of 0.9194, which indicated a good agreement between the experiment and the predicted values for biomass production.

- Linear coefficient B (pH), quadratic coefficients B^2 (pH) and C^2 (temperature) and interaction coefficients AB (salinity and pH) and AC (salinity and temperature) were significant model terms.
- The model implied that all the three factors such as salinity, pH and temperature had significant effect on biomass production.
- Using central composite design of response surface methodology the optimum growth conditions determined were NaCl content $^0/_{00}$, pH 6.51 and temperature 26.20ºC. In this set-up, the model predicted a maximum biomass concentration of 4.63gL^{-1}.
- The validation was carried out by shake flask experiment under optimum conditions of physical parameters predicted by the model. The experimental value for biomass production (4.53±0.005 gL^{-1}) were closer to the predicted value (4.63 gL^{-1}) validating the model. By using RSM for optimization of physical parameters, the yield was increased by 11.85% (4.05 ± 0.24 gL^{-1} to 4.53 ± 0.005 gL^{-1}) compared to biomass production under the laboratory conditions prior to optimization by RSM.
- Biomass production in fermenters was carried out in 5 L lab scale and 100 L pilot scale fermentors. The maximum yield attained was 7.4gL^{-1} and 11.54gL^{-1} (dry weight) respectively in 5 L lab and 100 L pilot scale fermenters.
- The mineral based media components such as maltose, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2$ and yeast extract were screened using Plackett-Burman Design (PBD). Thus maltose,

$MgSO_4$ and yeast extract were found to be significant medium components affecting biomass production.

- The significant medium components such as maltose, $MgSO_4$ and yeast extract were further optimized using FCCCD of RSM.
- The statistical significance of the second-order model equation was evaluated by the F-test analysis of variance which revealed that the regression was statistically significant ($P < 0.0004$) at 99% confidence level.
- The fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.9919, indicating that 99% of the variability in the response could be explained by the model. "Adj R^2" of 0.9847 and "Pred R^2" of 0.9548 reasonably agreed indicating a good conformity between the experimental and the predicted values for biomass production.
- Adequate precision obtained in this experiment was 13.323 which suggested that the model could be used to navigate the design space.
- The significance of the regression coefficients of the model indicated that the linear terms A (maltose), B ($MgSO_4$) and C (yeast extract), the quadratic terms A^2, B^2 and C^2 and interaction terms AC (maltose and yeast extract) and BC ($MgSO_4$ and yeast extract) were significant models values ($Prob>F< 0.05$) on biomass production. In this model maltose (A), $MgSO_4$ (B) and yeast extract (C) had a significant effect on biomass production by *Candida* MCCF 101.

- The optimum values given for maximum biomass productions were maltose - 50.8 gL^{-1}, $MgSO_4$- 1.8 $gL^{-1}$and yeast extract-18.0 gL^{-1}. In this set-up, the model predicted a maximum biomass concentration of 8.15gL^{-1}.
- The model was validated by repeating the experiment under optimum conditions in shake flask which resulted in 7.9gL^{-1} of biomass, very close to the predicted value of 8.15gL^{-1}.
- By using FCCCD for optimization of media ingredients, the yield could be increased from 3.89±0.09gL^{-1} to 7.9±0.07gL^{-1} compared to biomass production under the laboratory conditions prior to optimization by RSM.
- The maximum biomass production in fermentors attained was 16.06gL^{-1} in 5 L lab scale fermentor and 18gL^{-1}in 100 L pilot scale fermentor.
- The results obtained from the optimization of molasses based medium showed that the optimum concentration of the molasses was 3.5% for the better growth of *Candida* MCCF 101.
- Biomass production in molasses based medium was accomplished in 5L laboratory scale fermentor. The maximum biomass obtained in 72h was 5.8±0.28gL^{-1} (dry weight).
- A comparison of the mineral based medium and molasses based medium showed significant difference in biomass production.
- The maximum biomass production obtained in mineral based medium was 7.9±0.07 gL^{-1} and 16±0.20gL^{-1} in shake flask and laboratory scale fermentor respectively. In the case of molasses

based medium, it was 5.8±0.28gL^{-1} and 6.8±0.2gL^{-1} in shake flask and laboratory scale fermentor respectively. From this observation maximum biomass production could be found 2.35 fold higher in mineral based medium compared to that in molasses based medium.

- The growth performance and feed utilization of fish fed with diets containing different levels of dietary yeast concentrations of live and inactivated yeast preparations such as 10^6, 10^7, 10^8 and 10^9cfu g^{-1} were checked.
- Growth performance and feed utilization increased significantly ($P< 0.05$) by both live and inactivated yeast supplementation in the feed to Koi carp as evaluated by weight gain, SGR, FE, FCR, PI, PER.
- The fish fed with marine yeast isolate *Candida* MCCF 101supplemented diet weighed significantly more ($P< 0.05$) after 13 weeks of feeding.
- The maximum growth obtained on feeding with the optimum dietary live yeast concentration (10^6cfu g^{-1}) was 10.47±1.03g ($P< 0.05$) and 7.96±0.85g on feeding with inactivated yeast at 10^8cfu g^{-1} ($P< 0.05$). The lowest growth was obtained when fed on live yeast at 10^9cfu g^{-1} (4.07±0.33g) ($P> 0.05$) compared to the fishes fed on a diet not supplemented with yeast (control) (6.22±0.79g).
- Marine yeast *Candida* MCCF101 positively enhanced growth performance by weight gain, SGR, FE, FCR, PI and PER in Koi carp.

- When fed with diets containing live yeast 10^6cfu g^{-1}, Koi carp exhibited higher values of haematological and biochemical parameters such as RBCs, Hb, PCV, glucose, albumin, globulin and protein. On increasing the concentration of yeast beyond this level, the parameters showed a decreasing trend.
- Test of pathogenicity of *Aeromonas* sp. MCCB 113 in Koi carp as model organism determined by LD_{50} was $10^{7.1}$cfu mL^{-1}.
- The results showed that supplementation of yeast *Candida* MCCF 101 as live and inactive supplementation had a positive influence on the survival of Koi carp by resisting the *Aeromonas* sp. MCCB 113 infection.
- The highest percentage of survival was observed on feeding with live yeast at 10^6cfu g^{-1} (91.67%) followed by inactivated yeast at 10^7cfu g^{-1} (83.33%), which were higher than the other groups.
- Microscopic examination revealed that yeast cells were digested by Koi carp as intact cells were not seen.
- The marine yeast isolate, *Candida* MCCF 101 provided better nutritional and dietary values, increased biomass production, positively enhanced growth performance, immunocompetency and survival. It is concluded that *Candida* MCCF 101 is a potential strain to be used as feed supplement in aquaculture.

The future of single cell protein will be heavily dependent on reducing production costs and improving quality by fermentation, downstream processing and genetic improvement of producer organisms for enhancing the nutritional quality and biomass production. The selected

strains of yeast could be multiplied on cost effective raw materials using optimized culturing conditions followed by separation process. Elucidation of metabolic pathways and cell structure and applying latest innovations to make the product most suitable for aquaculture field are to be attempted.

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Appendices

Appendix -1

Gen bank submission

***Candida* sp. MCCF-101 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence**

GenBank: FJ652052.1

LOCUS FJ652052 511 bp DNA linear PLN 17-FEB-2009
DEFINITION Candida sp. MCCF-101 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ACCESSION FJ652052
VERSION FJ652052.1 GI:223551489
KEYWORDS.
SOURCE Candida sp. MCCF-101
ORGANISM Candida sp. MCCF-101
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida.
REFERENCE 1 (bases 1 to 511)
AUTHORS Poulose,S., Sarlin,P.J., Sajeevan,T.P., Philip,R. and Bright Singh,I.S.
TITLE Optimization of culture conditions, mass production and assessment of nutritional value of Candida MCCF 101 as shrimp feed supplement
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 511)
AUTHORS Poulose,S., Sarlin,P.J., Sajeevan,T.P., Philip,R. and Bright Singh,I.S.
TITLE Direct Submission
JOURNAL Submitted (21-JAN-2009) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin, Kerala 682016, India
FEATURES Location/Qualifiers
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5.8S          ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"
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    481 gctgaactta agcatatcaa taagcggagg a
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Appendix -2

Table 2.1 (a) ANOVA for the final weight of Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	64.936	4	16.234	22.485	0
Within Groups	7.22	10	0.722		
Total	72.156	14			

Table 2.1 (b) Homogenous subsets for the final weight of Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05		
	1	1	2	3
10^9	3	4.0667		
0	3	6.2	6.2	
10^8	3		7.4	
10^7	3		7.5667	
10^6	3			10.4667
Sig.		0.07	0.345	1

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.2 (a) ANOVA for the weight gain of Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	62.459	4	15.615	19.815	0
Within Groups	7.88	10	0.788		
Total	70.34	14			

Table 2.2 (b) Homogenous subsets for the weight gain of Koi carp fed with different groups of live yeast

Tukey HSD

Yeast con.(cfu g^{-1})	N	Subset for alpha = .05		
	1	1	2	3
10^9	3	3.1333		
0	3	5.1733	5.1733	
10^8	3		6.3333	
10^7	3		6.5667	
10^6	3			9.4
Sig.		0.104	0.366	1

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.3 (a) ANOVA for the specific growth rate of Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.32	4	0.33	8.953	0.002
Within Groups	0.369	10	0.037		
Total	1.688	14			

Table 2.3 (b) Homogenous subsets for the specific growth rate of Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05		
		1	2	3
10^9	3	1.6067		
0	3	1.9067	1.9067	
10^8	3	1.9733	1.9733	
10^7	3		2.1967	2.1967
10^6	3			2.4933
Sig.		0.21	0.399	0.379

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.4 (a) ANOVA for the feed efficiency of Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.06	4	0.015	6.328	0.008
Within Groups	0.024	10	0.002		
Total	0.084	14			

Table 2.4 (b) Homogenous subsets for the feed efficiency of Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^9	3	0.3	
0	3	0.39	0.39
10^7	3	0.4	0.4
10^8	3	0.4	0.4
10^6	3		0.5
Sig.		0.164	0.113

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.5 (a) ANOVA for the feed conversion ratio (FCR) of Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.069	4	1.017	3.062	0.069
Within Groups	3.322	10	0.332		
Total	7.392	14			

Table 2.5 (b) Homogenous subsets for the feed conversion ratio (FCR) of Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^6	3	2.2667
10^8	3	2.4333
0	3	2.8533
10^7	3	2.9
10^9	3	3.7667
Sig.		0.059

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000

Table 2.6 (a) ANOVA for the protein intake of Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.21	4	3.552	8.531	0.003
Within Groups	4.164	10	0.416		
Total	18.374	14			

Table 2.6 (b) Homogenous subsets for the protein intake of Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc. (cfu g^{-1})	N	Subset for alpha = .05		
	1	1	2	3
10^9	3	4.7667		
0	3	5.16	5.16	
10^8	3	5.2333	5.2333	
10^7	3		6.5667	6.5667
10^6	3			7.3333
Sig.		0.896	0.13	0.61

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.7 (a) ANOVA for the protein efficiency ratio (PER) of Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.948	4	0.237	4.09	0.032
Within Groups	0.579	10	0.058		
Total	1.527	14			

Table 2.7 (b) Homogenous subsets for the protein efficiency ratio (PER) of Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^9	3	0.6667	
0	3	1.0033	1.0033
10^7	3	1.0333	1.0333
10^8	3	1.2667	1.2667
10^6	3		1.4
Sig.		0.072	0.324

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.8 (a) ANOVA for the haemoglobin (Hb) levels in Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24.793	4	6.198	3.681	0.043
Within Groups	16.84	10	1.684		
Total	41.633	14			

Table 2.8 (b) Homogenous subsets for the haemoglobin (Hb) levels in Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc. (cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
0	3	4.3333	
10^8	3	4.9333	4.9333
10^7	3	5.5667	5.5667
10^9	3	6.5	6.5
10^6	3		8
Sig.		0.313	0.092

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.9 (a) ANOVA for the red blood cells (RBC) levels in Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.636	4	0.159	3.457	0.051
Within Groups	0.46	10	0.046		
Total	1.096	14			

Table 2.9 (b) Homogenous subsets for the red blood cells (RBC) levels in Koi carp fed with different groups of live yeast

Tukey HSD

Yeast con. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
0	3	0.6667
10^8	3	0.6667
10^7	3	0.7
10^9	3	1
10^6	3	1.1667
Sig.		0.098

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.10 (a) ANOVA for the white blood cells (WBC) levels in Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	285166.667	4	71291.667	2.935	0.076
Within Groups	242916.667	10	24291.667		
Total	528083.333	14			

Table 2.10 (b) Homogenous subsets for the white blood cells (WBC) in Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^9	3	283.3333
10^7	3	325
10^6	3	333.3333
10^8	3	383.3333
0	3	666.6667
Sig.		0.077

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000

Table 2.11 (a) ANOVA for the packed cell volume (PCV) in Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	299.984	4	74.996	2.613	0.099
Within Groups	287.06	10	28.706		
Total	587.044	14			

Table 2.11 (b) Homogenous subsets for the packed cell volume (PCV) in Koi carp fed with different groups live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^8	3	14
0	3	14.0667
10^7	3	16.6667
10^9	3	19.6667
10^6	3	26
Sig.		0.116

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.12 (a) ANOVA for the glucose level in Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3702	4	925.5	13.65	0
Within Groups	678	10	67.8		
Total	4380	14			

Table 2.12 (b) Homogenous subsets for the glucose levels in Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05		
	1	1	2	3
10^9	3	38.3333		
10^8	3	46	46	
0	3	48.3333	48.3333	
10^7	3		65.3333	65.3333
10^6	3			82
Sig.		0.592	0.095	0.172

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.13 (a) ANOVA for the albumin levels in Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.112	4	0.528	5.033	0.017
Within Groups	1.049	10	0.105		
Total	3.162	14			

Table 2.13 (b) Homogenous subsets for the albumin levels in Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^9	3	0.83	
10^7	3	1.3333	1.3333
10^8	3	1.3467	1.3467
10^6	3		1.8333
0	3		1.8367
Sig.		0.352	0.375

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.14 (a) ANOVA for the effect of globulin of Koi carp fed with live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.526	4	0.382	2.917	0.077
Within Groups	1.308	10	0.131		
Total	2.834	14			

Table 2.14 (b) Homogenous subsets for the globulin levels in Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^9	3	0.17
10^7	3	0.2333
10^8	3	0.52
0	3	0.7967
10^6	3	1
Sig.		0.105

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000

Table 2.15 (a) ANOVA for the protein level in Koi carp fed with different groups of live yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.897	4	1.724	13.196	0.001
Within Groups	1.307	10	0.131		
Total	8.204	14			

Table 2.15 (b) Homogenous subsets for the protein level in Koi carp fed with different groups of live yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^9	3	1	
10^7	3	1.5667	
10^8	3	1.8667	1.8667
0	3		2.6333
10^6	3		2.8333
Sig.		0.086	0.051

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.16 (a) ANOVA for the final weight of Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.54	4	2.135	2.37	0.122
Within Groups	9.009	10	0.901		
Total	17.549	14			

Table 2.16 (b) Homogenous subsets for the final weight of Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^9	3	6.2
0	3	6.22
10^6	3	6.3333
10^7	3	7.5667
10^8	3	7.9667
Sig.		0.228

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.17 (a) ANOVA for the weight gain of Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.11	4	2.528	5.463	0.014
Within Groups	4.627	10	0.463		
Total	14.737	14			

Table 2.17 (b) Homogenous subsets for the weight gain of Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc. (cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^6	3	4.8	
10^9	3	5.1	5.1
0	3	5.1733	5.1733
10^7	3	6.5	6.5
10^8	3		6.8333
Sig.		0.071	0.065

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.18 (a) ANOVA for the specific growth rate of Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.459	4	0.115	7.133	0.006
Within Groups	0.161	10	0.016		
Total	0.62	14			

Table 2.18 (b) Homogenous subsets for the specific growth rate of Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc (cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^6	3	1.77	
10^9	3	1.9	1.9
0	3	1.9067	1.9067
10^8	3		2.1733
10^7	3		2.2267
Sig.		0.686	0.062

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.19 (a) ANOVA for the feed efficiency of Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.025	4	0.006	5.949	0.01
Within Groups	0.01	10	0.001		
Total	0.035	14			

Table 2.19 (b) Homogenous subsets for the feed efficiency of Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^9	3	0.3	
10^6	3	0.3333	0.3333
0	3		0.39
10^7	3		0.4
10^8	3		0.4
Sig.		0.718	0.161

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.20 (a) ANOVA for the feed conversion ratio (FCR) of Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.03	4	0.258	3.011	0.072
Within Groups	0.855	10	0.086		
Total	1.886	14			

Table 2.20 (b) Homogenous subsets for the feed conversion ratio (FCR) of Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^8	3	2.6
10^6	3	2.7333
10^7	3	2.8
0	3	2.8533
10^9	3	3.3667
Sig.		0.057

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.21 (a) ANOVA for the protein intake of Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.001	4	1	1.796	0.206
Within Groups	5.571	10	0.557		
Total	9.572	14			

Table 2.21 (b) Homogenous subsets for the effect of protein intake of Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^6	3	5.1
0	3	5.16
10^8	3	6.0667
10^9	3	6.1
10^7	3	6.3333
Sig.		0.322

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.22 (a) ANOVA for the protein efficiency ratio (PER) of Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.127	4	0.032	3.413	0.052
Within Groups	0.093	10	0.009		
Total	0.219	14			

Table 2.22 (b) Homogenous subsets for the protein efficiency ratio (PER) of Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc (cfu g^{-1}).	N	Subset for alpha = .05	
	1	1	2
10^9	3	0.8333	
10^7	3	1	1
0	3	1.0033	1.0033
10^6	3	1.0667	1.0667
10^8	3		1.1
Sig.		0.083	0.713

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.23 (a) ANOVA for the haemoglobin (Hb) levels in Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.057	4	3.264	3.226	0.061
Within Groups	10.12	10	1.012		
Total	23.177	14			

Table 2.23 (b) Homogenous subsets for the haemoglobin (Hb) levels in Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast con. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^6	3	4.2333
0	3	4.3333
10^7	3	4.8
10^8	3	6
10^9	3	6.5667
Sig.		0.1

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.24 (a) ANOVA for the red blood cells (RBC) levels in Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.139	4	0.035	1.441	0.29
Within Groups	0.242	10	0.024		
Total	0.381	14			

Table 2.24 (b) Homogenous subsets for the red blood cells (RBC) levels in Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast con. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
0	3	0.6667
10^6	3	0.7833
10^7	3	0.8
10^8	3	0.8333
10^9	3	0.9667
Sig.		0.203

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.25 (a) ANOVA for the white blood cells (WBC) levels in Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	499166.667	4	124791.667	7.114	0.006
Within Groups	175416.667	10	17541.667		
Total	674583.333	14			

Table 2.25 (b) Homogenous subsets for the white blood cells (WBC) levels in Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast con. (cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^9	3	166.6667	
10^7	3	200	
10^8	3	266.6667	
10^6	3	408.3333	408.3333
0	3		666.6667
Sig.		0.243	0.195

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.26 (a) ANOVA for the packed cell volume (PCV) levels in Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	295.496	4	73.874	2.981	0.073
Within Groups	247.853	10	24.785		
Total	543.349	14			

Table 2.26 (b) Homogenous subsets for the packed cell volume (PCV) levels in Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^6	3	12.2
0	3	14.0667
10^7	3	14.3
10^8	3	19
10^9	3	24.4667
Sig.		0.076

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.27 (a) ANOVA for the glucose levels in Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1952.267	4	488.067	33.277	0
Within Groups	146.667	10	14.667		
Total	2098.933	14			

Table 2.27 (b) Homogenous subsets for the glucose levels in Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc.(cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^9	3	40.3333	
0	3	48.3333	
10^6	3		63.6667
10^8	3		66.3333
10^7	3		70
Sig.		0.153	0.321

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.28 (a) ANOVA for the albumin levels in Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.513	4	0.628	6.896	0.006
Within Groups	0.911	10	0.091		
Total	3.425	14			

Table 2.28 (b) Homogenous subsets for the albumin levels in Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast con. (cfu g^{-1})	N	Subset for alpha = .05	
	1	1	2
10^6	3	1.4	
10^7	3	1.4233	
10^9	3	1.5967	
0	3	1.8367	1.8367
10^8	3		2.51
Sig.		0.438	0.118

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.29 (a) ANOVA for the globulin levels in Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.912	4	0.228	1.093	0.411
Within Groups	2.085	10	0.209		
Total	2.997	14			

Table 2.29 (b) Homogenous subsets for the globulin levels in Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast conc. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^8	3	0.19
10^9	3	0.57
10^6	3	0.7667
0	3	0.7967
10^7	3	0.8767
Sig.		0.403

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table 2.30 (a) ANOVA for the protein levels in Koi carp fed with different groups of inactivated yeast

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.789	4	0.197	0.628	0.653
Within Groups	3.14	10	0.314		
Total	3.929	14			

Table 2.30 (b) Homogenous subsets for the protein levels in Koi carp fed with different groups of inactivated yeast

Tukey HSD

Yeast con. (cfu g^{-1})	N	Subset for alpha = .05
	1	1
10^6	3	2.1667
10^9	3	2.1667
10^7	3	2.3
0	3	2.6333
10^8	3	2.7
Sig.		0.77

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.