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
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth of 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
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

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,
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

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; Rodriguez 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, MgSO₄·7H₂O-1.8gL⁻¹, and yeast extract-18gL⁻¹) with pH 6.51 and incubated at 26.3°C 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 10⁶, 10⁷, 10⁸ and 10⁹ cfu 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°C 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.
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

until feed consumption and general behavior became normal. Water temperature ranged from 27°C to 29°C, dissolved oxygen concentrations from 4.3 to 6.7 mgL⁻¹, pH from 7.2 to 8.0, and unionized ammonia concentration from 0.04 to 0.14 mgL⁻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 into nine groups. One was kept as control and the other eight groups included treatment with live and inactivated yeast at concentrations 10⁶, 10⁷, 10⁸ and 10⁹ 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, NH₃-N, NO₂-N and NO₃-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 0.1 ppm.

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 10⁶, 10⁷, 10⁸ and 10⁹ cfu g⁻¹.
Chapter 5

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 = $W_2 - W_1$

Percentage of weight gain = wt. gain/ $W_1 \times 100$

Specific growth rate (SGR) = $100 \frac{\ln W_2 - \ln W_1}{T}$;  
(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) $\times 100$

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

Total protein intake (PI) = feed intake (g) $\times$ 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°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°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 intraperitoneal (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 $1 \times 10^4$, $1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$ 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
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

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 ($1 \times 10^7$ cfu 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
Chapter 5

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°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, K₂HPO₄ - 1.0g, NH₄H₂PO₄ - 1.0g, MgSO₄.7H₂O - 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 28⁰C. 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 Leifson's basal medium was used for this purpose, which contained, Peptone - 2.0g, NaCl - 5.0g, K₂HPO₄ - 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±0.5⁰C 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
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth...

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 $10^6$ cfu g$^{-1}$ (10.47 ± 1.03 g) (p < 0.05) followed by inactivated yeast at $10^8$ cfu g$^{-1}$ (7.96 ± 0.85 g) (p < 0.05).

However, low growth was observed when fed on live yeast at an elevated concentration of $10^9$ cfu g$^{-1}$ (4.07 ± 0.33 g) (p > 0.05) compared with the batch of fishes fed on the diet not supplemented with yeast (6.22 ± 0.79 g). Moreover, when fishes were fed with diets containing live yeast at the concentration of $10^6$ cfu g$^{-1}$ and inactivated yeast at $10^8$ cfu 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 $10^9$ cfu 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 $10^6$ cfu g$^{-1}$ (1.30 ± 0.28) and $10^8$ cfu g$^{-1}$ (1.26 ± 0.43) followed by inactivated yeast at $10^8$ cfu g$^{-1}$ (1.12 ± 0.08) had the highest PER; the lowest PER was observed with fishes fed on live yeast at $10^9$ cfu g$^{-1}$ (0.66 ± 0.04). Fishes fed with live yeast at $10^6$ cfu 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 \(10^6\text{cfu g}^{-1}\) \((7.83 \pm 0.20 \text{g dL}^{-1})\) and followed by inactivated yeast at \(10^6\text{cfu g}^{-1}\) \((7.5 \pm 0.20 \text{g dL}^{-1})\). 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 \((666 \pm 230.9 \times 10^3 \text{µL}^{-1})\). 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 \(10^6\text{cfu g}^{-1}\) 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 \(10^6\text{cfu g}^{-1}\) \((82 \pm 16.64 \text{mg dL}^{-1})\) which got reduced significantly in the group fed with live yeast at \(10^9\text{cfu g}^{-1}\) \((38.3 \pm 1.53 \text{mg dL}^{-1})\) compared to that of the control \((48.3 \pm 6.66 \text{mg dL}^{-1})\). Serum protein level registered slight increase in the batches of fishes fed on live yeast at \(10^6\text{cfu g}^{-1}\) \((2.83 \pm 0.29 \text{g dL}^{-1})\) and exhibited significant reduction in the batches fed with live yeast at \(10^9\text{cfu g}^{-1}\) \((1 \pm 0.17 \text{g dL}^{-1})\) compared to the one recorded in the control \((2.63 \pm 0.38 \text{g dL}^{-1})\). Albumin levels were significantly influenced by Candida MCCF 101 supplement, as the lowest level was observed in the fishes fed on live yeast at \(10^9\text{cfu g}^{-1}\) \((0.83 \pm 0.06 \text{g dL}^{-1})\) and the highest in the fishes fed with inactivated yeast at \(10^8\text{cfu g}^{-1}\).
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

(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 10⁶cfu g⁻¹ to 10⁹cfu g⁻¹ compared with that of the control group. Yeast supplementation increased glucose, globulin and protein values higher in live yeast fed groups at 10⁶cfu g⁻¹.

5.3.3 Challenge study

5.3.3.1 LD₅₀ of *Aeromonas* sp. MCCB 113

The virulence of *Aeromonas* MCCB 113 was assessed *in vivo* from the LD₅₀ value. Koi carp (*Cyprinus carpio haematopterus*) was used as the test model and LD₅₀ of the *Aeromonas* sp. MCCB 113 was found to be 10⁷.1 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 10⁶cfu g⁻¹ (91.67%) followed in the group fed on inactivated yeast at 10⁷cfu g⁻¹ (83.3%), whereas, the lowest survival was obtained in the group of fishes fed with live yeast at 10⁸cfu 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.
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

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 $10^6$ cfu g$^{-1}$ followed by inactivated yeast at $10^8$ cfu g$^{-1}$ as dietary feed supplements enhanced the growth of koi carp, whereas live and inactivated yeast concentration of $10^9$ cfu g$^{-1}$ depressed the growth, even below 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 (10^6 cfu 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 10^9 cfu 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 10^6 cfu g\(^{-1}\). Protein
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

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 $10^6$cfu g$^{-1}$ (1.30±0.28) and inactivated yeast at $10^8$cfu 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 $10^9$cfu 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 $10^6$cfu g$^{-1}$ exhibited higher RBCs, Hb, PCV, glucose, albumin, globulin and protein values. However, on feeding with increased concentration of yeast above $10^6$cfu 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 *Candida* 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 $10^6$cfu g$^{-1}$ (91.67%) followed by inactivated yeast fed at $10^7$cfu 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 $10^8$cfu g$^{-1}$ and $10^9$cfu 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,
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

*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 \(10^6\text{cfu g}^{-1}\) and \(10^8\text{cfu g}^{-1}\) 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

<table>
<thead>
<tr>
<th>Items</th>
<th>Control</th>
<th>Live yeast (cfu/mL⁻¹)</th>
<th>10⁶</th>
<th>10⁷</th>
<th>10⁸</th>
<th>10⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Initial wt (g)</td>
<td>1.03±0.06ᵃ</td>
<td>1.08±0.04ᵃ</td>
<td>1.02±0.05ᵇ</td>
<td>1.03±0.23ᵇ</td>
<td>0.94±0.03ᵇ</td>
<td></td>
</tr>
<tr>
<td>2 Final wt(g)</td>
<td>6.22±0.79ᵇ</td>
<td>10.47±1.03ᵃ</td>
<td>7.57±0.92ᵇ</td>
<td>7.38±0.92ᵇ</td>
<td>4.07±0.33ᶜ</td>
<td></td>
</tr>
<tr>
<td>3 Weight gain(g)</td>
<td>5.19±0.81ᵇ</td>
<td>9.39±1.05ᵃ</td>
<td>6.55±0.92ᵇ</td>
<td>6.35±1.15ᵇ</td>
<td>3.13±0.32ᶜ</td>
<td></td>
</tr>
<tr>
<td>4 Specific growth rate(g)</td>
<td>1.97±0.17ᵇ</td>
<td>2.49±0.13ᵃ</td>
<td>2.20±0.15ᵇ</td>
<td>1.98±0.36ᵇ</td>
<td>1.61±0.09ᶜ</td>
<td></td>
</tr>
<tr>
<td>5 Feed efficiency</td>
<td>0.39±0.04ᵇ</td>
<td>0.46±0.10ᵃ</td>
<td>0.35±0.02ᵇ</td>
<td>0.45±0.15ᵇ</td>
<td>0.26±0.02ᵇ</td>
<td></td>
</tr>
<tr>
<td>6 FCR</td>
<td>2.85±0.30ᵃ</td>
<td>2.27±1.50ᵃ</td>
<td>2.91±0.67ᵇ</td>
<td>2.42±0.90ᵇ</td>
<td>3.80±0.24ᵃ</td>
<td></td>
</tr>
<tr>
<td>7 Protein intake (%)</td>
<td>5.16±0.50ᵇ</td>
<td>7.33±0.78ᵃ</td>
<td>6.59±0.49ᵇ</td>
<td>5.23±0.91ᵇ</td>
<td>4.77±0.45ᶜ</td>
<td></td>
</tr>
<tr>
<td>8 PER</td>
<td>1.0±0.10ᵇ</td>
<td>1.30±0.28ᵃ</td>
<td>1.00±0.21ᵇ</td>
<td>1.26±0.43ᵇ</td>
<td>0.66±0.04ᵇ</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Items</th>
<th>Control</th>
<th>Inactive yeast (cfu/mL⁻¹)</th>
<th>10⁶</th>
<th>10⁷</th>
<th>10⁸</th>
<th>10⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Initial wt (g)</td>
<td>1.03±0.06ᵃ</td>
<td>1.12±.34ᵃ</td>
<td>1.04±0.16ᵃ</td>
<td>1.13±0.01ᵃ</td>
<td>1.1±0.14ᵃ</td>
<td></td>
</tr>
<tr>
<td>2 Final wt(g)</td>
<td>6.22±0.79ᵇ</td>
<td>6.33±1.60ᵃ</td>
<td>7.54±0.34ᵃ</td>
<td>7.96±0.85ᵃ</td>
<td>6.2±0.76ᵃ</td>
<td></td>
</tr>
<tr>
<td>3 Weight gain(g)</td>
<td>5.19±0.81ᵇ</td>
<td>5.38±1.37ᵇ</td>
<td>6.49±0.20ᵇ</td>
<td>6.84±0.85ᵃ</td>
<td>5.1±0.66ᵇ</td>
<td></td>
</tr>
<tr>
<td>4 Specific growth rate(g)</td>
<td>1.97±0.17ᵇ</td>
<td>1.91±0.42ᵇ</td>
<td>2.18±0.12ᵃ</td>
<td>2.14±0.11ᵃ</td>
<td>1.90±0.9ᵇ</td>
<td></td>
</tr>
<tr>
<td>5 Feed efficiency</td>
<td>0.39±0.04ᵇ</td>
<td>0.36±0.04ᵇ</td>
<td>0.36±0.01ᵃ</td>
<td>0.39±0.03ᵃ</td>
<td>0.30±0.0ᵇ</td>
<td></td>
</tr>
<tr>
<td>6 FCR</td>
<td>2.85±0.30ᵃ</td>
<td>2.76±0.27ᵃ</td>
<td>2.80±0.04ᵃ</td>
<td>2.56±0.19ᵃ</td>
<td>3.34±0.47ᵃ</td>
<td></td>
</tr>
<tr>
<td>7 Protein intake (%)</td>
<td>5.16±0.50ᵃ</td>
<td>5.07±0.96ᵃ</td>
<td>6.32±0.24ᵃ</td>
<td>6.08±0.32ᵃ</td>
<td>6.09±1.19ᵃ</td>
<td></td>
</tr>
<tr>
<td>8 PER</td>
<td>1.0±0.10ᵇ</td>
<td>1.05±0.11ᵇ</td>
<td>1.03±0.01ᵇ</td>
<td>1.12±0.08ᵇ</td>
<td>0.85±0.11ᵇ</td>
<td></td>
</tr>
</tbody>
</table>

Mean values having the same superscript in the same row are not significantly different at P≤0.05.
The marine isolate *Candida MCCF 101* as dietary feed supplement to enhance growth.

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

<table>
<thead>
<tr>
<th>Items</th>
<th>Control</th>
<th>Live yeast (cfu mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10⁶</td>
</tr>
<tr>
<td>HB (g/dL)</td>
<td>4.8 ± 0.61b</td>
<td>7.83 ± 0.20a</td>
</tr>
<tr>
<td>RBC (10⁹/µL)</td>
<td>0.72 ± 0.18a</td>
<td>1.14 ± 0.20a</td>
</tr>
<tr>
<td>WBC (10⁷/µL)</td>
<td>666 ± 230.9a</td>
<td>333.35 ± 7.7a</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>14 ± 2.80a</td>
<td>25.76 ± 2.86a</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>48.3 ± 6.66bc</td>
<td>82 ± 16.64a</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>1.84 ± 0.29a</td>
<td>1.83 ± 0.29a</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>0.8 ± 0.60a</td>
<td>1 ± 0.50a</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Items</th>
<th>Control</th>
<th>Inactive yeast (cfu mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10⁶</td>
</tr>
<tr>
<td>HB (g/dL)</td>
<td>4.8 ± 0.61a</td>
<td>4.05 ± 2.34a</td>
</tr>
<tr>
<td>RBC (10⁹/µL)</td>
<td>0.72 ± 0.18a</td>
<td>0.8 ± 0.46a</td>
</tr>
<tr>
<td>WBC (10⁷/µL)</td>
<td>666 ± 230.9a</td>
<td>325 ± 256.6ab</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>14 ± 2.80a</td>
<td>12.2 ± 7.16a</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>48.3 ± 6.66bc</td>
<td>63.7 ± 1.53a</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>1.84 ± 0.29ab</td>
<td>1.4 ± 0.36b</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>0.8 ± 0.60a</td>
<td>0.77 ± 0.31a</td>
</tr>
<tr>
<td>Protein (g/dL)</td>
<td>2.63 ± 0.38a</td>
<td>2.17 ± 0.57a</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Bacterial Dose</th>
<th>Infected test units</th>
<th>Cumulative infected (A)</th>
<th>Cumulative non infected (B)</th>
<th>Ratio A/A+B</th>
<th>% of infected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1×10⁸</td>
<td>8/8</td>
<td>13</td>
<td>0</td>
<td>13/13</td>
<td>100</td>
</tr>
<tr>
<td>1×10⁷</td>
<td>4/8</td>
<td>5</td>
<td>4</td>
<td>5/9</td>
<td>55.5</td>
</tr>
<tr>
<td>1×10⁶</td>
<td>1/8</td>
<td>1</td>
<td>11</td>
<td>1/12</td>
<td>8.33</td>
</tr>
<tr>
<td>1×10⁵</td>
<td>0/8</td>
<td>0</td>
<td>11</td>
<td>0/11</td>
<td>0</td>
</tr>
<tr>
<td>1×10⁴</td>
<td>0/8</td>
<td>0</td>
<td>11</td>
<td>0/11</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0/8</td>
<td>0</td>
<td>11</td>
<td>0/11</td>
<td>0</td>
</tr>
</tbody>
</table>

LD₅₀ = 10⁷.¹ CFU/mL

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

<table>
<thead>
<tr>
<th>Phenotypic characteristic</th>
<th><em>A.Caviae</em></th>
<th>T-1</th>
<th>T-2</th>
<th>T-3</th>
<th>T-4</th>
<th>T-5</th>
<th>T-6</th>
<th>T-7</th>
<th>T-8</th>
<th>T-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram's stain</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MOF</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Kovac's oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O/129 sensitivity</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. DL- lactate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2. D-Cellobiose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3. Salicine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.
Fig. 1(a-c) Over view of feeding experimental setup
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

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

"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"
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

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

"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"
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

Fig.4 (a-d) Lethal dose (LD₅₀) determination by intraperitoneal (IP) injection and the experimental set up
Chapter 5

**Fig. 5** Yeast supplementation as evaluated by weight gain

**Fig. 6** Percentage survival of Koi carp after challenge with *Aeromonas* sp. MCCB 113
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

(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)
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

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

(a) Light microscopy

(b) Dark field microscopy

(c) Phase contrast microscopy
Chapter 5

(a) Light microscopy

(b) Dark field microscopy

(c) Phase contrast microscopy

Fig. 10 (a-c) Positive controls (feed with inactivated yeast)
The marine isolate Candida MCCF 101 as dietary feed supplement to enhance growth.

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

(a) Light microscopy

(b) Dark field microscopy

(c) Phase contrast microscopy
Chapter 5

(a) Light microscopy

(b) Dark field microscopy

(c) Phase contrast microscopy

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

.....SIGNS.....