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

3.1 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 (e.g. 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:

\[
\text{Absorbance} = \log \left( \frac{I_o}{I} \right)
\]

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 into 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
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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 and 11.0 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°C (Barnett *et al.*, 1990). Some psychrophilic yeast grows optimally at temperatures between 12 and 15°C. Higher temperatures in the range of 30-37°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°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, Kloechera 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°C and 20-25°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).
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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

\[ \eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2; \]

\[ \text{--------------------------------------------}(1) \]

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:

\[ \eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2; \]

\[ \text{--------------------------------------------}(2) \]
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 \( \eta \) is the estimated response, \( \beta_0 \) is a constant, \( \beta_1, \beta_2, \beta_{11}, \beta_{22}, \beta_{12} \), are the coefficients for each term and \( x_1, x_2 \) and \( x_3 \) are the independent variables.

OR

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j, \quad \text{(4)} \]

Where \( Y \) represents the response variable, \( \beta_0 \) represents the interception coefficients, \( \beta_i \) is the coefficient of linear effect, \( \beta_{ii} \) is the coefficient of quadratic effect and \( \beta_{ij} \) 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.
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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:

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

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 20 parts 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 (1.39×10⁵ cfu mL⁻¹), 0.05 (2.79×10⁵ cfu mL⁻¹), 0.10 (5.57×10⁵ cfu mL⁻¹), 0.15 (8.44×10⁵ cfu mL⁻¹), 0.2 (11.14×10⁵ cfu mL⁻¹) and incubated at 25.0 ±1°C 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 \((5.57 \times 10^5 \text{cfu mL}^{-1})\) 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 40°C in a temperature controlled rotary shaker.
Optimization of physical parameters such as pH, temperature and NaCl content in malt from (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°C. 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. ²³ = 8 factorial points, 2 x 3 = 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:

$$Y = b_0 + b_1.X_1 + b_2.X_2 + b_3.X_3 + b_{12}.X_1X_2 + b_{23}.X_2X_3 + b_{13}.X_1X_3 + b_{11}.X_1^2 + b_{22}.X_2^2 + b_{33}.X_3^2$$  \hspace{1cm} (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°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°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°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 (1.39x10⁵cfu mL⁻¹) to 0.2
(11.4×10^5 cfu mL\(^{-1}\)). Hence the cell suspension having absorbance 0.1 at Abs\(_{540}\) (5.57×10^5 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)

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

(Where ‘Y’ is the predicted biomass and A, B and C are the coded values of NaCl content, pH and temperature respectively).
Optimization of physical parameters such as pH, temperature and NaCl content in malt ….

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 (R²) which could also be expressed in percentage and was interpreted as the percentage variability in the response in the given model. R² 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 R²” of 0.7283 was in reasonable agreement with the “Adj R²” 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 26.3°C. 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±0.005gL\(^{-1}\)) 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 ± 0.24gL\(^{-1}\) to 4.53 ± 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°C and maximum production was observed at 25°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°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 \textit{Candida} MCCF 101, NaCl content of 20g\textit{L}\(^{-1}\) 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 \textit{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 0.0g\textit{L}\(^{-1}\) 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\(^{\circ}\)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.
Optimization of physical parameters such as pH, temperature and NaCl content in malt ….

Table 1 Experimental range and levels of the independent variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Code</th>
<th>Range studied</th>
<th>Range and levels</th>
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</thead>
<tbody>
<tr>
<td>Salinity (gL⁻¹)</td>
<td>A</td>
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<td>0 25 50</td>
</tr>
<tr>
<td>pH</td>
<td>B</td>
<td>3-7.5</td>
<td>3.5 5.5 7.5</td>
</tr>
<tr>
<td>Temperature(ºC)</td>
<td>C</td>
<td>20-35</td>
<td>20 27.5 35</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Salinity (gL⁻¹)</th>
<th>Significance</th>
<th>pH</th>
<th>Significance</th>
<th>Temperature(ºC)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>0.036</td>
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</tr>
<tr>
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<tr>
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"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"
Table 3  Central Composite Design (CCD) matrixes of the three variables along with the experimental and predicted values of *Candida* MCCF 101.

<table>
<thead>
<tr>
<th>Expt.No</th>
<th>NaCl (gL⁻¹)</th>
<th>pH</th>
<th>Temperature(°C)</th>
<th>Biomass (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Experimental</td>
</tr>
<tr>
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<td>5.5</td>
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<td>25</td>
<td>5.5</td>
<td>27.5</td>
<td>4.5</td>
</tr>
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<td>5.5</td>
<td>27.5</td>
<td>4.01</td>
</tr>
<tr>
<td>17</td>
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<td>5.5</td>
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<td>4.03</td>
</tr>
<tr>
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<td>5.5</td>
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<td>20</td>
<td>25</td>
<td>5.5</td>
<td>27.5</td>
<td>4.05</td>
</tr>
</tbody>
</table>
Optimization of physical parameters such as pH, temperature and NaCl content in malt.

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

"Marine yeast Canafila MCCF 161 as feed supplement in aquaculture: nutritional quality, optimization of large scale production and evaluation of its protective effect on Koi carp from Aeromonas infection"
Fig. 3 Yeast cells separated by centrifugation in heavy duty centrifuge.

Fig. 4 Effective of inoculum on biomass production.
Optimization of physical parameters such as pH, temperature and NaCl content in malt ....

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

(b)

"Marine yeast Candida MCF 161 as feed supplement in aquaculture: nutritional quality, optimization of large scale production and evaluation of its protective effect on Koi carp from Aeromonas infection"
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.
Fig. 7(a-b) Biomass production in laboratory scale fermentor (a) and pilot scale fermentor (b).
Fig. 8 Packed single cell protein