Chapter 3

Optimisation of the culture conditions of *Candida sake* for biomass production
3.1. Introduction

All organisms need a variety of elements as nutrients like carbon, nitrogen, phosphorous trace elements etc. for growth. In nature these nutrients are dispersed among large number of compounds, which are either inorganic or organic forms. Carbon is one of the most important elements required for microbial growth. Heterotrophs require preformed organic compounds like carbohydrates, amino acids, peptides and organic acids. All wild type yeasts utilize glucose, mannose, fructose, sucrose etc as carbon source. Yeasts generally utilize ammonium salt as sole source of nitrogen (Jones et al., 1981). Diammonium phosphate is utilized more preferentially than ammonium chloride (Spencer et al., 1997). Some strains of yeast utilize urea as source of nitrogen apart from ammonium ions. Other elements like hydrogen, oxygen, sulphur and phosphorus also seem to be essential for microbial growth.

Thus a good culture medium should contain all these essential nutrients needed for the growth at optimal level. Not only the optimal nutritional requirements but also the physico-chemical parameters like temperature, salinity and pH should be taken care of while designing a culture medium for mass cultivation of a microorganism. Cost reduction by utilizing less expensive substrates seems to be an economically viable option. In this chapter attempt was made to utilize cane sugar molasses as the sole carbon source. Other nutritional requirements and physicochemical parameters were optimised by considering one-factor-at-a time for marine yeast Candida sake S165.

3.2. Materials and methods

3.2.1. Preparation of inoculum

Yeast inoculum was prepared by harvesting young culture (2 days old growth) of yeast Candida sake S165 into sterile seawater of salinity 15ppt.
Optical density of the culture suspension was measured at 540 nm and adjusted to 0.1 OD using sterile seawater. 10 μl of this suspension was used as inoculum in 10 ml culture medium for the following optimisation processes.

3.2.2. Optimisation of salinity
Molasses based culture medium of nine different salinities like 0, 5, 10, 15, 20, 25, 30, 35 and 40 ppt. were prepared and sterilized. 10 μl inoculum was added to each medium and incubated for 48hrs at room temperature and growth was measured turbidometrically using Hitachi 2001 UV series spectrophotometer at 540nm.

3.2.3. Optimisation of pH
To find out the pH optima, molasses based seawater (15ppt) medium with pH of 3, 3.5, 4, 4.5, 5, 5.5, 6, 7 and 8 were prepared. The pH of the medium was adjusted either by using 0.1N NaOH or 0.1N HCl solution. 10 μl each of the cell suspension was added to the medium (10 ml) and after 48 hrs of incubation, growth was measured at 540nm.

3.2.4. Optimisation of temperature
10 μl inoculum of Candida sake was added to 10ml molasses based seawater (15ppt) medium and incubated at different temperatures like 20, 25, 27, 30, 35, 40 and 45°C and growth was measured at 540nm after 48 hrs of incubation.

3.2.5. Optimisation of carbon source
Sugar cane molasses, the main by-product from the sugar industry, can be used after proper dilution as a cheap carbon source for fermentation process as it is rich in sugar, mostly in the form of sucrose, fructose and glucose. Sugar cane molasses, obtained from a private distillery located at Cherthala, was diluted with distilled water (100gm in 200ml distilled water) and used as stock solution, the total sugar of which was determined by Anthrone method.

Optimisation of the culture conditions of Candida sake for biomass production
A Marine Isolate Candida sake as source of immunostimulants to Fenneropenaeus indicus

(Roe, 1955). Molasses medium with varying concentrations of total sugar viz. 0.5, 1, 2, 3, 4 and 5 mg/ml were prepared using the stock solution. After inoculation with 10μl yeast suspension, incubation was done at room temperature (28 ± 2°C) for 48 hrs and the growth was measured at 540nm.

3.2.6 Optimisation of nitrogen source
Three different compounds KNO₃, Urea and (NH₄)₂SO₄ were tested as a source of nitrogen for yeast. Molasses based seawater (15ppt) medium with five different concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5%, of each of these three compounds were prepared and inoculated with 10 μl yeast suspension of 0.1 OD. After incubation at room temperature (28 ± 2°C) for 48 hrs, growth was measured at 540nm.

3.2.7 Optimisation of phosphorus source
As source of phosphorus KH₂PO₄ was used at different concentrations like 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5% in molasses based seawater (15ppt) medium and 10 μl yeast inoculum was added to 10 ml culture medium. Cultures were incubated at room temperature (28 ± 2°C) for 48 hrs and growth was measured at 540nm.

3.2.8 Optimisation of magnesium
MgSO₄·7H₂O was used as the source of magnesium in which different concentrations, 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05% were tested to find out the optimum concentration required for growth. 10 ml molasses based seawater (15ppt) medium were prepared and inoculation of 10 μl yeast suspension of 0.1 OD was done. After incubation at room temperature (28 ± 2°C) for 48 hrs growth was measured at 540nm.

3.2.9 Optimisation of calcium
Different concentrations of CaCO₃ like 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5% were tested to find the optimal concentration required for growth of C. sake. After inoculation with 10μl yeast suspension in molasses based seawater (15ppt)
medium, incubation was done at room temperature (28 ± 2°C) for 48 hrs and the growth was measured at 540nm.

3.3. Results

Growth was found to be maximum at 15 ppt salinity. However, C. sake exhibited good growth rate at 0 to 35 ppt. (Fig. 3.1). Candida sake was found to grow at a wide range of pH from 3 to 8 without showing much difference in growth. The optimum growth was found to be at pH 4.5 (Fig 3.2). Growth was found to be maximum at 30°C and it remained more or less the same between the room temperature (28±2°C) to 35°C. (Fig. 3.3). Beyond this range considerable reduction in growth could be observed.

Molasses concentration was expressed as total sugars (mg/ml medium). A concentration of 2mg/ml was found to be optimal for the growth of Candida sake (Fig. 3.4). However no significant change in growth could be observed when the concentration was between 0.5 and 3 mg/ml. Concentration above 3 mg/ml resulted in reduced growth.

Ammonium sulphate was found to be the most preferred nitrogen source. Even though growth was found to be maximum with 0.3% (NH₄)₂SO₄, no significant variation in growth could be observed at a concentration range of 0.1 to 0.5% (NH₄)₂SO₄ (Fig. 3.5). With urea as nitrogen source at 0.1 and 0.2% the growth was found to be almost equal to that of (NH₄)₂SO₄ medium. However, higher concentrations of urea resulted in drastic reduction in growth. Growth was uniformly lesser at all concentrations of KNO₃ (0.1 to 0.5%) compared to the other two nitrogen sources.

Growth was found to be maximum with 0.3% K₂HPO₄ in the medium (Fig 3.6). A considerable increase in growth could be observed when K₂HPO₄ concentration in the medium was increased from 0.5 to 0.3% and a gradual reduction was noticeable beyond this level.
Growth was found to be almost at the same level with 0.02 to 0.05% MgSO₄ concentrations in the medium, the maximum being at 0.05% (Fig 3.7). At 0.2% CaCO₃ concentration the growth of *C. sake* was found to be maximum (Fig 3.8). A gradual reduction in growth could be observed with increased concentration of CaCO₃ in the medium.

3.4. Discussion

The indigenous nature of marine yeasts is not yet clear, whether they are true inhabitants of the sea or merely transient forms introduced from freshwater or terrestrial habitats. *Candida sake* S165 used in the present study was found to be growing optimally at 15 ppt and comparatively good growth could be observed at 0 to 35 ppt salinity without showing much variation. This shows the ability of the particular yeast strain to survive both in fresh water and seawater conditions. Kriss (1963) reported that the majority of yeasts in the sea are not accidental forms, but species adapted to the life under marine conditions. The present study also supports this observation.

Other physical parameters for growth like temperature and pH show that *C. sake* S 165 prefer 30°C and pH 4.5. Yeasts generally prefer an acidic pH for growth, which would be advantageous to prevent bacterial contamination during mass production, where absolute sterility cannot be ensured. Furlan et al. (2001) also reported that lower pH was important for better cell growth of yeast *Kluyveromyces marxianus*. Anas and Singh (2003) reported that yeast *Acremonium dyospori* preferred pH 4 for higher cell yield. In the present work, temperature preference of *C. sake* was 30°C for optimal growth, which was perfectly suitable for mass cultivation in a tropical country like India were the ambient atmospheric temperature is similar to this optimum value.

The development of an economically viable culture medium is necessary to obtain high quantity of biomass. Carbon substrate has a dual role in
biosynthesis and energy generation, with carbohydrates being the usual carbon source for microbial fermentation process (Stanbury et al., 1995). The most widely available carbohydrate is starch obtained from maize grains, other cereals, potatoes and cassava. However, majority of the published papers deal with supplementation with compounds such as yeast extract, a highly effective, but expensive material, which enhances the biomass production. Costa et al. (2002) reported that soluble starch provided good growth yields when yeast extract was used as a nitrogen source for *Pantoea agglomerans*. Therefore, from aquaculture point of view the chance of using such an uneconomical material appears unlikely. Consequently there is a need for more extensive investigation on the utilization of cheap raw materials as supplements for yeast biomass production, in particular, increased attention has to be paid to the possibility of using by-products and waste materials from the food industry. A number of unrefined carbon sources have been utilized in fermentation industry by various workers; cane molasses (Haard, 1988), sugar cane juice (Fontana et al., 1996), corn wet milling co-products (Hayman et al., 1995) and grape juice (Meyer and Du Preez, 1994).

Cane sugar molasses is an abundant by-product of the sugar industry, only partially used in fermentative processes and its disposal still remains a problem (Chiarini et al., 1992). Many workers have reported the utilization of molasses as a source of carbon in fermentation processes (Oderinde et al., 1990; Ergun et al., 1997; Furlan et al., 2001). Aksu and Kutsal (1986) studied the lactic acid production by *Lactobacillus delbrueckii* by using beet molasses as carbon source. Goksungur et al. (2002) reported that beet molasses solution supplemented with yeast extract and corn steep liquor was an attractive medium for the production of β-carotene. In the present study *C. sake* grew optimally in medium containing molasses as a carbon source (total sugars 2mg/ml). The higher concentrations of molasses did not show any increase in SCP production, which may be due to the hyperosmotic environment of the medium. Jones et al. (1981) had opined that
A Marine isolate *Candida sake* as source of immunostimulants to *Fenneropenaeus indicus*

higher sugar concentration in culture medium probably inhibits fermentation where plasmolysis of yeast cells could occur. Bajaj *et al.* (2003) reported loss of viability of yeast strains in molasses medium due to higher osmotic pressure of medium with reduced water activity and inhibitory substances of molasses.

The nitrogen content of yeast is about 10% of dry weight and as such it represents an important constituent of any growth medium. Yeast generally utilize ammonium salt as sole source of nitrogen and diammonium phosphate is utilized most efficiently and ammonium chloride least whereas some strain can even utilize urea (Spencer *et al.*, 1997). Complex nitrogen sources such as peptone, yeast extract and tryptone, contain nitrogenous fractions like amino acid, peptides, nucleic acids etc. in an undefined proportion. In the present study, \((\text{NH}_4\text{)}_2\text{SO}_4\) at a concentration of 0.3% was found to be optimum for growth and proved to be a better nitrogen source for commercial production of yeast biomass. A similar observation was made by Khan *et al.*(1995) who reported that \((\text{NH}_4\text{)}_2\text{SO}_4\) was a suitable nitrogen source for the production of yeast biomass. Incorporation of urea in media at concentration of 0.1 to 0.2% was found to support good growth and the lesser growth at higher concentrations could be due to its toxic effect.

Yeasts utilize inorganic phosphates for growth. It is taken up as the monovalent anion, \(\text{H}_2\text{PO}_4^-\), and more is taken up as monobasic potassium salt than the dibasic sodium form (Spencer *et al.*, 1997). Present experiment showed that *C. sake* preferred 0.3% \(\text{KH}_2\text{PO}_4\) in culture medium.

Metal ions such as \(\text{Mg}^{2+}\), \(\text{Ca}^{2+}\) and \(\text{K}^+\) directly influence fermentation metabolism in yeast (Gamarallage *et al.*, 1997). Magnesium and potassium are regarded as bulk cations establishing the required ionic environment of the cell, and magnesium transport by yeast has been reported to be dependent on the presence of potassium (Bahadur and Verma, 1959; Borst-Pauwells, 1981). In the present study supplementation of molasses medium
with 0.03% magnesium in the form of MgSO$_4 \cdot 7$H$_2$O showed optimum growth for Candida sake. Similarly the CaCO$_3$ supplementation at a concentration of 0.2% showed optimum growth and higher concentrations induced slight decrease in cell growth. Kotzamanidis et al., (2002) reported a similar observation in the lactic acid production from beet molasses by using Lactobacillus delbrueckii where reduced cell growth was observed due to high concentration (7% w/v) of CaCO$_3$. Calcium carbonate controls the pH of the fermentation medium. Tanaka and Omura (1986) reported calcium carbonate as the most common buffering agent used in fermentation experiments. Supplementation of all other micronutrients appears to be not necessary if seawater is used for media preparation, where seawater contains all these compounds and other trace elements.
Fig. 3.1 Effect of salinity on the growth of *Candida sake*

Fig. 3.2 Effect of pH on the growth of *Candida sake*
Fig. 3.3 Effect of temperature on the growth of *Candida sake*

Fig. 3.4 Effect of molasses on the growth of *Candida sake*
Fig. 3.5 Effect of nitrogen sources on the growth of *Candida sake*. Data with same superscript do not vary significantly ($P<0.05$).

Fig. 3.6 Effect of phosphorus on the growth of *Candida sake*.
Fig. 3.7 Effect of magnesium on the growth of *Candida sake*

Fig. 3.8 Effect of calcium on the growth of *Candida sake*