Original Article

A MOLASSES BASED FERMENTATION MEDIUM FOR MARINE YEAST BIOMASS PRODUCTION

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Abstract

Four marine yeasts were used for the study based on their performance in a feeding experiment in *Fenneropenaeus indicus* viz., 1. *Debaryomyces hansenii* (S8) 2. *Debaryomyces hansenii* (S100), 3. *Candida sake* (S165) and 4. *Candida tropicalis* (S186). Molasses was the most preferred carbon source by the marine yeasts compared to glucose, sucrose and rice water. Molasses (amount of total sugars 9mg/ml) supplemented with peptone (0.75%), yeast extract (0.5%) and MgSO\(_4\) (0.25%) was found to be favouring maximum growth of the four yeast strains tested. Two yeast strains (S8 & S186) showed their maximum growth at 30ppt salinity and the other two (S100 & S165) at 25ppt and 20ppt respectively. pH 6 was found to be most favourable for growth. This study shows that molasses supplemented with peptone and yeast extract could be used as a good production medium for large scale production of yeast biomass.

Key words: Marine yeast, molasses, fermentation medium, salinity, pH.

1. Introduction

Complex nutrients are preferred in fermentation media as they often support higher yields; and chemically defined media are rarely used due to economic reasons. Of all the medium components used, carbon and nitrogen sources are of particular importance in the medium, since microbial cells are composed largely of these elements. The major carbon and nitrogen source of fermentation media are soybean meal, molasses, corn steep liquor, sulphite waste liquor, cotton seed meal, yeast extract, peptone etc. Calcium chloride, ammonium phosphate and potassium phosphate are incorporated for enhanced growth. Microbes grow more vigorously on complex media than in mineral media, because the former contain biosynthetic precursors that can be channeled directly into anabolic pathways reducing the need to produce them and saving metabolic energy. Pharmamedia, molasses, corn steep liquor, sulphite waste liquor are used as fermentation substrates for microbes. [1] tested the optimal growth condition of two marine yeast strains *D. hansenii* (Yeast-14) and *C. austromarina* (Yeast-16) and observed that maximal growth was in the range of 20 - 25°C respectively.

The marine yeasts displayed maximum optical density in the pH range 3-8 and showed an abrupt decline in growth at NaCl concentration above 5% level. An incubation temperature of 20-25°C is often used for the growth of mesophilic yeasts [2, 3]. [4] studied the influence of pH and temperature on the growth and citric acid production of *Yarrowialipol* with glucose as a substrate and found that the best fermentation conditions were pH 6 and temperature 30°C. *Debaryomyces nepalensis*, a halo tolerant food spoiling yeast could grow in complex (YEPD) medium at pH 3.0 to 11.0 in the absence of salt and pH 3.0-9.0 in the presence of different concentrations of NaCl and KCl [5]. *Debaryomyces hansenii* is salt tolerant and it has been reported that some strains are able to grow in the presence of 20% (W/V) NaCl [6] and *Zygosaccharomyces rouxii* is recognized for its tolerance of high concentrations of sugars [7]. *Debaryomyces hansenii*, and its anamorph *Candida formata*, are cryotolerant marine yeasts which can tolerate salinity levels up to 24%, whereas *Saccharomyces cerevisiae* growth is inhibited when salinity reaches 10%. The present study is focused on the selection of a cheap and
suitable substrate for the production of marine yeast biomass for application in food and feed industry.

2. Materials and methods

2.1 Yeasts used for the study

Four marine yeasts (Fig.1) were used for the study based on their performance in feeding experiment in *Fenneropenea indicus* viz., 1. *Debaryomyces hansenii* (S8) 2. *Debaryomyces hansenii* (S100) 3. *Candida sake* (S165) and 4. *Candida tropicalis* (S186). These yeast strains were originally isolated from the Arabian Sea and maintained in the Microbiology Laboratory of the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology.

Fig.1. Marine yeasts used for the study (100x).

2.2 Preparation of inoculum

The yeast isolates were streaked onto malt extract agar (malt extract, 30g; mycological peptone, 5g; Agar-Agar, 20g; sea water, 1Litre; pH, 7.6) slants and after incubation for 3-4 days at room temperature (28±2°C), the cells were harvested using sterile physiological saline (0.9% NaCl in distilled water). The optical density of the cell suspension was adjusted to 10D (approximately 4X10⁸ cells/ml) and this cell suspension was used as inoculum. 1 ml was inoculated to 100 ml medium.

2.3. Measurement of growth

The yeast growth was estimated by measuring the optical density at 540nm in a Hitachi Model 200-20 UV Visible Spectrophotometer.

2.4 Selection of a suitable substrate as Carbon source for growth

Four different media (M₁, M₂, M₃ and M₄) were prepared incorporating substrates such as glucose, sucrose, rice water and molasses as Carbon sources. The basal medium (KNO₃, 2g; Seawater, 1000ml; pH-5.5) was supplemented with Glucose, 20g (M₁); Sucrose, 20g (M₂); Rice water, 100ml (M₃); and Molasses, 50ml (M₄). 1ml aliquots of the inoculum were transferred into 100ml media. Incubated at room temperature (28±2°C), for 48 hours and the growth were measured by recording the optical density at 540 nm in a Hitachi Model 200-20 UV-Visible spectrophotometer. Based on the results medium containing molasses (M₄) was selected for further study (Fig. 2).

Fig.2 Growth of marine yeasts in media with different C sources ( M₁ - Glucose , M₂ - Sucrose , M₃ – Rice water and M₄ - Molasses).

2.5 Optimization of the concentration of molasses in medium.

Concentration of molasses in the medium was expressed in terms of total sugars estimated by Anthrone Method [8]. 50g of molasses in 100ml seawater was used as the stock. Media with different concentrations of molasses (1 to 10mg/ml total sugars) were prepared. After sterilization, the selected marine yeasts were inoculated and they were incubated at room temperature (28±2°C) for 48 hours and growth was estimated at 540nm.

2.6 Optimum peptone concentration

Molasses media with different peptone concentration (0, 0.25, 0.5, 0.75 and 1%) were prepared. Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540nm.

2.7 Optimum yeast extract concentration

Molasses media (M₄) with different yeast extract concentration (0, 0.25, 0.5, 0.75 and 1%) were prepared. Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540nm.

2.8 Optimum Magnesium sulphate concentration

Molasses media (M₄) supplemented with different concentrations of magnesium sulphate (0, 0.25, 0.5, 0.75 and 1%) were prepared. Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540nm.

2.9 Optimum Calcium chloride concentration

Molasses media with different calcium chloride concentrations (0, 0.25, 0.5, 0.75 and 1%) were prepared. Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540nm.

2.10 Optimum Potassium dihydrogen phosphate concentration

Molasses media supplemented with different concentrations of potassium dihydrogen phosphate (0, 0.1, 0.2, 0.3 and 0.4%) were prepared. Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540nm.

2.11 Effect of salinity

Molasses medium (M₄) was prepared in seawater of different strength (0, 10, 15, 20, 25, 30, 35 and 40 ppt).
Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540nm.

2.12 Effect of pH
Molasses media (M₄) with different pH (4,5,6,7,8,9 and 10) were prepared using various buffers. (Various buffers used were Sodium acetate-acetic acid pH-5.0, Tris-Maleic acid pH-6.0-7.0, Tris-HCl Buffer pH-8.0, NaHCO₃-Na₂CO₃ pH-9.0). All the four yeast strains were inoculated and incubation was done at room temperature (28±2°C) for 48 hours.

3. Results
3.1 Selection of suitable Carbon source for media preparation
Molasses was found to be the best carbon source supporting maximum growth followed by rice water and sucrose. Maximum growth was exhibited by Debaryomyces hansenii (S100) followed by Candida sake (S165). Growth in molasses medium was found to be almost double of that found in the other media.

3.2 Effect of molasses concentration on growth
Growth of marine yeasts was found to be influenced by the concentration of molasses in the medium. Maximum growth was observed at a concentration of total sugars 9mg/ml for all the four strains tested. A gradual increase in growth could be observed with the increase of molasses concentration. However, the presence of total sugars more than 9mg/ml was found to have adverse effect and resulted in lesser growth (Fig.3).

3.3 Supplementation of Peptone and Yeast extract
Even though a significant increase in growth could not be observed with the supplementation of peptone in the medium, maximum growth could be obtained at a concentration of 0.75% (Fig. 4). Considerable increase in growth could be observed when yeast extract was introduced into the medium and the growth was found to be maximum at 0.5% (Fig. 5).

Fig. 3. Effect of molasses concentration on the growth of yeast strains. Data are given as mean ± SD, n=3.

Fig. 4. Growth of yeast strains at various peptone concentrations in the molasses medium. Data are given as mean ± SD, n=3.

Fig. 5. Growth of yeast strains at various yeast extract concentrations in the molasses medium. Data are given as mean ± SD, n=3.

Fig. 6. Growth of yeast strains at various magnesium sulphate concentrations in the molasses medium. Data are given as mean ± SD, n=3.

Fig. 7. Growth of yeast strains at various calcium chloride concentrations in the molasses medium. Data are given as mean ± SD, n=3.
3.4 Supplementation of Magnesium, Calcium and Potassium

Generally growth was found to be maximum at a concentration of 0.25% MgSO$_4$ in the medium (Fig.6). The optimal calcium chloride concentration for growth was found to be 0.15% for all the strains (Fig.7). Generally the optimum potassium dihydrogen phosphate concentration was 0.3% (Fig.8).

![Fig. 8. Growth of yeast strains at various KH$_2$PO$_4$ concentration in the molasses medium. Data are given as mean± SD, n=3.](image)

3.5 Effect of salinity and pH on growth

A salinity of 30ppt was preferred by S8 and S186 whereas S100 showed maximum growth in 25ppt and S165 in 20ppt (Fig.9). Growth was meager at pH 4 and increased considerably at pH 5 reaching the maximum at pH 6 (Fig.10).

![Fig. 9. Effect of salinity on the growth of selected strains. Data are given as mean ± SD, n=3](image)

![Fig. 10. Effect of pH on the growth of selected yeast strains. Data are given as mean ± SD, n=3.](image)

4. Discussion

Molasses was found to be the most preferred substrate by the marine yeasts for growth and a concentration of 9mg/ml (total sugars) was found to be optimal for all the four strains. *S. cerevisiae* had the highest cell viability and ethanol production in a molasses medium containing 25% (w/v) total sugars at 35°C [9]. Molasses being a waste from sugar industry, its utilization would be highly economical for SCP production. Molasses as a crude C source might have contributed better growth due to the presence of other nutrients. The results obtained with rice water was not very promising displaying the nutritional insufficiency of the medium. In the present study, besides the carbon sources supplied, the medium contained only KNO$_3$ (N source) and the nutrients present in the seawater. [10] has reported that Cane molasses used as C source contain 60% sucrose in addition to growth promoting components. Cane molasses (3.5-17.5% w/v total sugar) and yeast powder (1.5-5% w/v) were used in the formulation of media for the cultivation of *Aspergillus japonicus* FCL-119T and *Aspergillus niger* ATCC 20611 [11]. They found that lower sugar concentration produced lower cellular growth. Carbon sources in fermentation media can be simple or complex carbohydrates, sugar alcohols or other alcohols, organic acids, proteins, peptides, amino acids and even hydrocarbons. These carbon sources are usually used in a crude form of sugars which include beet and cane molasses, corn molasses, whey, sulfite waste liquor, cull fruits, cannery wastes and so forth. Polysaccharides such as starches are supplied by corn, wheat, rye, milo, rice, potatoes, sweet potatoes and other agricultural products. Cellulosic byproducts also are usable as carbon sources, but they usually require costly saccharification by a procedure such as acid hydrolysis. Hydrocarbon substrates are usually a mixture of various hydrocarbon components and are relatively inexpensive. The pure hydrocarbon compounds or hydrocarbon fractions, however, are more costly. Several of the better crude nutritive sources for fermentation media are themselves complex mixtures of nutrients, supplying carbon and nitrogen compounds as well as microbial growth factors. Specific examples are molasses, corn steep liquor and sulfite waste liquor. The overall composition of the various molasses differs according to the specific geographic areas of production. Beet and Cane molasses are by-products of the sugar industry and different names are applied to the molasses depending on the particular step from which it was recovered. Of these, blackstrap molasses prepared from sugar cane normally is the cheapest and the most used sugar source for industrial fermentations. Beet molasses are produced by procedures resembling those for sugar cane. However, beet molasses may be limiting in biotin for yeast growth so that a small amount of cane blackstrap or other source of biotin should be added for growth of these microorganisms. Hydrol is a molasses resulting from the manufacture of crystalline dextrose from corn starch. It contains approximately 60% sugar, but it also contains a relatively high salt concentration that must be considered if this molasses is to be used as a medium component. Cane molasses is the final run-off syrup from sugar manufacture.
Total residual sugars in molasses can amount to 50-60% (w/v), of which 60% is sucrose. In addition to sucrose there are both growth promoting components [10] and inhibitors, e.g. Hydroxymethyl furfural [12].

### 4.1 Supplementation of Peptone and Yeast extract

The optimum peptone concentration was found to be 0.75%. Generally for bacteria a concentration of 0.5 to 1% is incorporated in media. In the present study also, the optimal concentration falls within this range. The optimum yeast extract concentration for growth was found to be 0.5% for all the four strains. [13] reported that the maximum production of cephalosporin C by *Acremonium chrysogenum* was achieved by employing wheat rava with 1% soluble starch and 1% w/w yeast extract at 30°C. Yeast growth medium require more amount of yeast extract in it than that required in bacteriological media. ZoBell’s agar contains only 0.1% yeast extract, whereas GPY (Glucose Peptone Yeast extract) used for yeast cultivation contain 1% yeast extract. The presence of hydrolyzed yeast components would be definitely supporting good growth.

### 4.2 Supplementation of Calcium, Magnesium and phosphate

The optimum magnesium sulphate concentration was found to be 0.25%. [14] observed that the optimal medium for pullulan production by *Rhodotorula bacular* was 8.0% (w/v) glucose, 2.0% (w/v) soybean cake hydrolysate, 0.5% (w/v) MgSO₄·7H₂O, and 0.06% (NH₄)₂SO₄, pH 7.0. Calcium chloride can control pH of the fermentation medium and has its’ own effect on growth. In the present investigation, presence of 0.15% calcium chloride in the medium was most favourable for growth.

Phosphate can enhance or suppress the production of growth at different concentrations. According to [15] vegetative growth of yeasts increased with the initial phosphate concentration up to 5mM, a further increase of phosphate showed no significant effect on cell yield. In the present study the optimum phosphate concentration was found to be in the range 0.2 to 0.3%.

### 4.3 Effect of salinity and pH

Among the four strains used for the study, two strains (S8 and S18) showed 30ppt as their optimal salinity for growth. Other two strains (S100 and S165) showed maximum growth at 25ppt and 20ppt respectively. Strains which showed a preference for 30ppt seawater exhibit their halophilic nature. *D. hansenii* is salt tolerant and it has been reported that some strains are able to grow in the presence of 20% (w/v) NaCl [6, 16]. Similar observations were made by [17] that the *D. hansenii* can grow under low water activity. The greater tolerance of *Debaryomyces hansenii* and *Saccharomyces hansenii* to NaCl at pH 5.0 – 7.0 compared with other pH values has also been reported by Hobot and [18]. *Debaryomyces hansenii* is cryotolerant marine yeast which can tolerate salinity levels up to 24%. An optimal pH of 6 was found to be very much favourable for growth by majority of the strains. Environmental pH is particularly significant in determining the growth of yeasts [19, 20]. Most yeasts grow within the range of pH 3.0-7.0 [21, 22, 23]. The effects of temperature, pH and NaCl concentration on the growth of 13 strains of yeasts representing five genera: *Debaryomyces*, *Pichia*, *Zygosaccharomyces*, *Candida* and *Saccharomyces* revealed that there was a synergistic effect between NaCl and pH at lower temperatures. The pH of seawater is about 7.8 and therefore marine yeasts usually prefer a higher pH for growth compared to those isolated from fruits and terrestrial environments, which prefer a pH of 4.5-5.5 for maximal growth [24]. The optimum process variables that supported maximum phytase production by a marine yeast *Kodamaea ohmeri* G3 were statistically determined as 23.0% NaCl and initial pH 6.3 [25].

[5] noticed that the growth of the food spoilage yeast *D. nepalensis* in NaCl and KCl was completely inhibited when temperature was maintained at extreme conditions (-1 and +1). At extreme pH of 3.6, specific growth was 0.29 and 0.37/h for NaCl and KCl, respectively exhibiting the yeast’s preference for acidic conditions for growth. Optimization of the process parameters using RSM clearly showed the strong interaction effect between the pH, temperature, and salt concentration on the growth of food spoilage yeast *D. nepalensis*.

[26] found that a strain of *Candida* sp. S27 acts as a good immunostimulant and antioxidant when applied as a dietary supplement in *Penaeus monodon*. [27] observed that *Candida* sp. S27 promote growth in *Penaeus monodon* while supplied through diet at 5% level. Molasses being a byproduct from sugar industry is a cheap source of Carbon for fermentation industry. Marine yeast prefers molasses for its growth and thereby mass production of yeast single cell protein can be achieved by using molasses as the basal medium. Supplementation of Nitrogen source and minerals enhanced the yeast biomass production. Molasses supplemented with peptone, yeast extract and minerals can be used as a good fermentation medium for large scale production of yeast biomass for application in food and feed industry.

### References


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