



## OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION OF SINGLE CELL PROTEIN FROM MARINE YEAST *CANDIDA* MCCF 101 AS FEED SUPPLEMENT IN AQUACULTURE

Sunitha Poulose and Bright Singh, I. S.\*

National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Lakeside Campus, Fine Arts Avenue, Cochin – 682016

\*Corresponding author: [isbsingh@gmail.com](mailto:isbsingh@gmail.com)

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**Abstract:** This study was undertaken to optimize the most important growth parameters for the development of an appropriate bioprocess for large scale production of the marine yeast *Candida* MCCF 101 as feed supplement in aquaculture, as it had proven immunostimulatory properties in shrimp. In the present study, using shake flask experiments the growth conditions optimized for biomass production were NaCl concentration  $20\text{gL}^{-1}$ , pH 4.5 and temperature  $25^{\circ}\text{C}$ . However, the organism is capable to grow in a range of 0 -  $100\text{gL}^{-1}$  NaCl. Among the various carbon sources investigated the highest biomass yield of *Candida* MCCF 101 was obtained by incorporating maltose as single carbon source and  $\text{NH}_4\text{Cl}$  as the nitrogen source. The experimental data revealed that the *Candida* MCCF 101 had significant biomass production at a wide range of maltose (2-5%),  $\text{NH}_4\text{Cl}$  (0.025-0.3%),  $\text{KH}_2\text{PO}_4$  (0.2-0.6%),  $\text{MgSO}_4$  (0.030-0.070%),  $\text{CaCl}_2$  (0.0125-0.025%) and yeast extract (0.01-0.06%). It is concluded that the marine yeast *Candida* MCCF 101 can be mass produced in a medium composed of maltose 2%,  $\text{NH}_4\text{Cl}$  0.1%,  $\text{KH}_2\text{PO}_4$  0.5%,  $\text{MgSO}_4$  0.06%,  $\text{CaCl}_2$  0.015%, yeast extract 0.03%, NaCl  $20\text{gL}^{-1}$ , pH 4.5 and temperature  $25^{\circ}\text{C}$ .

**Key words:** biomass, shake flask experiment, growth parameters, physical factors, media components

### INTRODUCTION

Aquaculture is a rapidly growing industry recognized as a viable and profitable enterprise world over (Tacon, 1999). In aquaculture nutrition it becomes absolutely essential to supply feed to meet the nutritional requirements for enhanced growth and productivity (De Silva, 1993). Increase in fish biomass in culture systems become increasingly depended on the supplementary feed for all nutrients (Lovell, 1998). Feed supplements are expected to provide proteins, lipids, carbohydrates and energy as well as vitamins and micro and macro nutrients to enable fish to remain free from stress and diseases (Nakagawa *et al.*, 2010). In this context microbes as feed supplements produced through biotechnological processes have been actively investigated as alternative or unconventional feed supplements for

aquaculture and aquaculture systems (Banerjee *et al.*, 2000). Yeast is commonly used in aquaculture, either as live feed organisms, or after processing, as a feed ingredient (Stones and Mills, 2004). Yeast and yeast derivatives are natural diet additives that have been shown as effective growth enhancers and immunostimulants (Oliva-Teles and Goncalves, 2001; Ozorio *et al.*, 2010; Andrews *et al.*, 2011) of some fish species and shrimps (Burgents, 2004). Yeasts are good sources of vitamins B, E, and D. SCP seems to be a potential source of protein and is playing a greater role in the evolution of aquaculture especially to fish and crustacean (Nayar *et al.*, 1998; Ricci *et al.*, 2003). Many species of yeasts used as sources of SCP are *Pichia*, *Candida*, *Saccharomyces*, *Kluyveromyces*, *Torulopsis*, *Hansenula*, *Koloechera* etc. Sajeevan *et al.* (2009) observed

that marine yeast biomass can be directly added to the larval rearing system as feed supplement for better protection and survival against microbial infection. Marine yeast have been regarded as safe and showing a beneficial impact on biotechnological process. It provides better nutritional and dietary values indicating their potential application as feed supplements in aquaculture.

All possible conventional methods for the production of SCP should be thoroughly examined for their possible economic application. All the factors such as pH, temperature, humidity, oxygen requirement, carbon and nitrogen sources ought to be optimized thoroughly before using the production on a large scale. Production process development is carried out in three distinct steps, (i) Process development using shake flasks, (ii) Scaling up using small to medium fermentors and (iii) Large scale production. One factor at a time method can be applied for optimization of medium components as well as for process condition and it is based on the classical method of one independent variable while fixing all others at a certain level (Alexeeva *et al.*, 2002; Paditar *et al.*, 2005; Ahamed *et al.*, 2006). This strategy has the advantage that it is simple, easy and the individual effects of the medium components and process condition can be seen on graphs (Kar *et al.*, 1999; Kumar *et al.*, 2003). Therefore, the present study was undertaken, and focused on laboratory level process development of production of marine isolate *Candida* MCCF 101 using shake flasks experiment following the principle of one factor at a time.

## MATERIALS AND METHODS

### Yeast Strain

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

India as *Candida* MCCF 101. The yeast was cultured in seawater based malt extract broth (Salinity 20gL<sup>-1</sup>) composed of (g L<sup>-1</sup>) malt extract 17g and mycological peptone 3g.

### Culture techniques

Twenty four hour old lawn culture of the yeast was harvested into sterile seawater of salinity 20gL<sup>-1</sup>. Its absorbance was adjusted to 0.1 at 540nm using sterile sea water to obtain a cell count of 5.57×10<sup>5</sup> which was used as the inoculum for further experiments.

### Shake flask experiment

The optimization of growth conditions was carried out in Erlenmeyer flasks (250ml) with 50ml mineral based medium broth.

### Optimization of physical factors for yeast biomass production

#### Optimization of NaCl content

Culture broth was prepared at 50 ml aliquots at different salinities such as 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 by addition of NaCl (g L<sup>-1</sup>) to distilled water. The flasks were inoculated with 0.1ml cell suspension having an absorbance of 0.1 at Abs540 nm and incubated at 28±1°C for 72 hours 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).

#### Optimization of pH

Culture broth was prepared in 50 ml aliquots in saline water (20 gL<sup>-1</sup>) 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.

#### Optimization of Temperature

Culture broth prepared in 50 ml aliquots in saline water (20 gL<sup>-1</sup>) 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 (Orbitek - Scigenics Biotech. (Pvt) Ltd, Chennai, India) at 120 rpm. Growth was measured using spectrophotometer at Abs 540nm.

## Screening of carbon and nitrogen sources

### Selection of carbon source

Carbon source was varied in the first set of experiments. Different carbon sources used were glucose (1%), sucrose (1%), starch (1%), xylose (1%), lactose (1%), and maltose (1%). The experiments were carried out in 250 mL Erlenmeyer flasks with a working volume of 50 mL. The flasks were inoculated with 0.1mL cell suspension and incubated at 26.3°C for 72 hours on a rotary shaker at 100rpm and growth was measured from the absorbance at Abs540nm in a spectrophotometer (UV-1601, Shimadzu Corporation, Tokyo, Japan).

### Selection of nitrogen source

The nitrogen source was altered in this set of experiments. Nitrogen sources selected were  $(\text{NH}_4)_2\text{SO}_4$  (1%),  $\text{KNO}_3$  (1%),  $\text{NH}_4\text{Cl}$  (1%),  $(\text{NH}_4)_2\text{HPO}_4$  (1%) and  $\text{NH}_4\text{NO}_3$  (1%). The experiments were carried out in 250mL Erlenmeyer flasks containing 50 mL medium. Flasks were inoculated as above and incubated at 26.3°C for 72 hours. Growth was measured at Abs540nm.

### Optimization of media ingredients in mineral based medium

Mineral based media with different concentrations of maltose (1.0-5.0%),  $\text{NH}_4\text{Cl}$  (0.02-5.0%),  $\text{KH}_2\text{PO}_4$  (0.05-0.60%),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.00-0.07%),  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  (0.00-0.06%) and yeast extract (0-0.07%) were prepared and inoculated with *Candida* MCCF 101. The concentration of one ingredient was varied over the given range, while, all the other ingredients were kept constant at their standard levels. Growth was measured at Abs540nm.

### 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 (5g $\cdot$ L $^{-1}$ NaCl), re-suspended in fresh saline and measured absorbance 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).

## RESULTS

### Shake flask experiment

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

### Optimization of physical factors

One dimensional screening was undertaken to find out the optimum value of each physical parameter such as NaCl content, pH, and temperature. Accordingly, NaCl content, 20g $\cdot$ L $^{-1}$ , pH, 4.5 and temperature 25°C (Fig.1a-c) were the optimised for further analysis.

### Selection of carbon and nitrogen sources

### Optimization of carbon and nitrogen sources

A number of different carbon and nitrogen sources were tested and biomass production monitored during the experiment. Among the different substrates tested as carbon source,

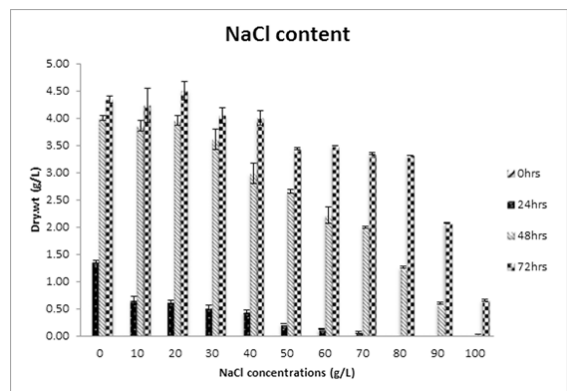


Fig. 1. a

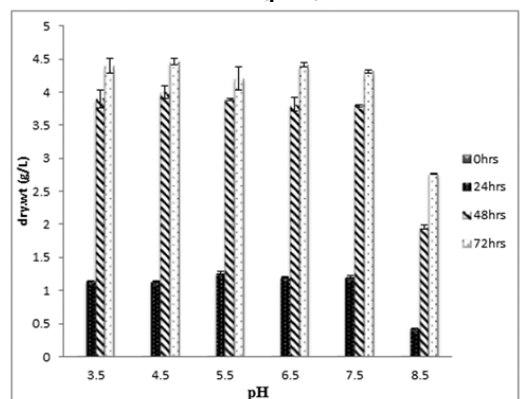
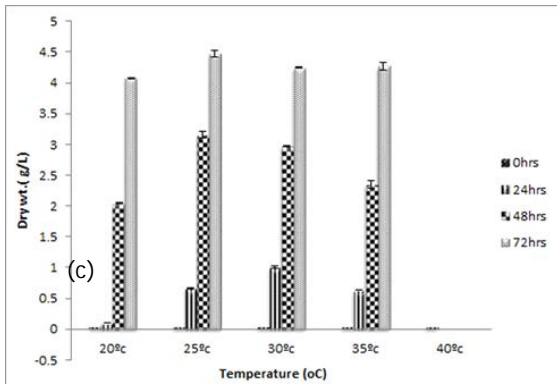
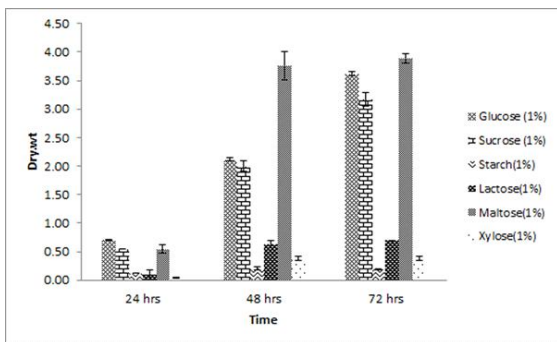


Fig. 1. b

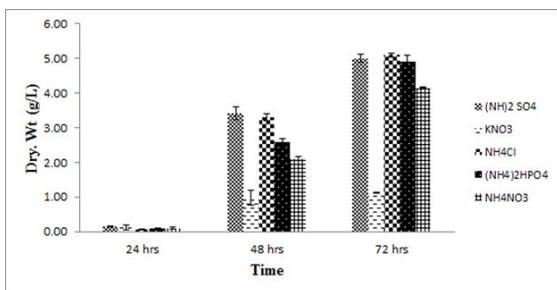
# Optimization of culture conditions for the production of SCP from marine yeast



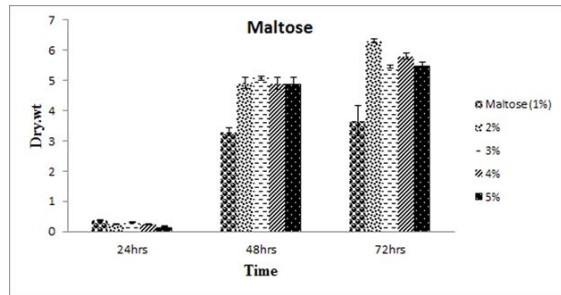
**Figs. 1 (a-c)** One dimensional screening of physical factors such as NaCl content, pH and temperature affecting biomass production



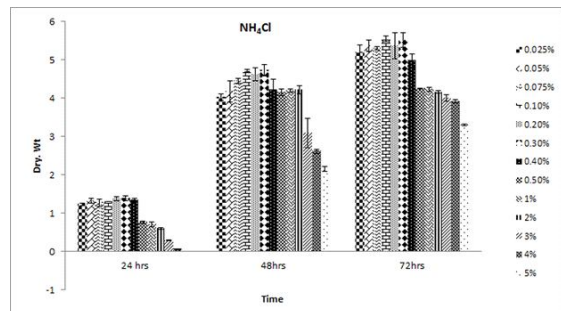
**Fig. 2.** Effect of carbon source on biomass production of *Candida* MCCF 101



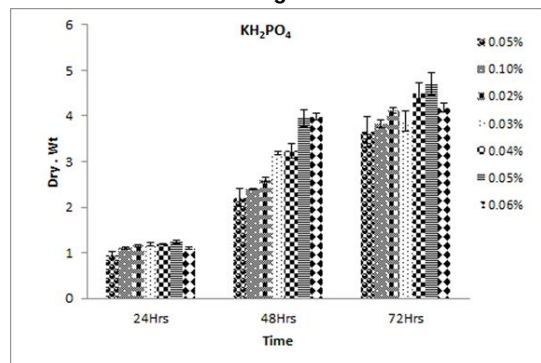
**Fig. 3.** Effect of nitrogen source on biomass production of *Candida* MCCF 101



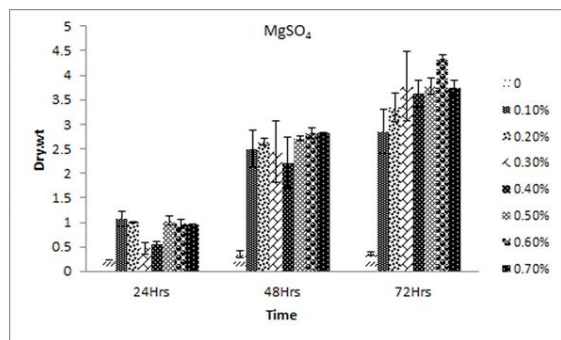
**Fig. 4. a**



**Fig. 4. b**



**Fig. 4. c**



**Fig. 4. d**

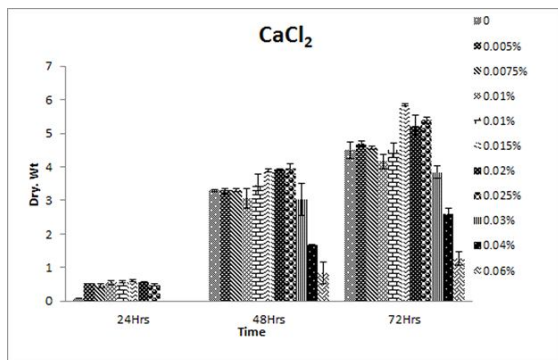


Fig. 4. e

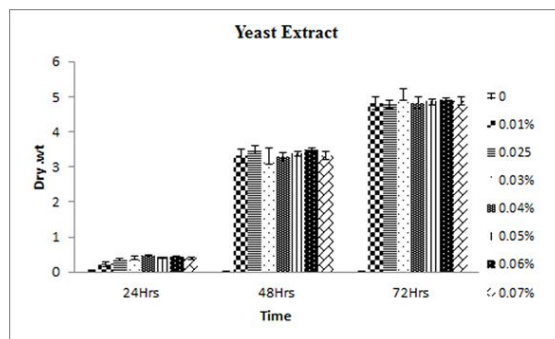


Fig. 4. f

Figs. 4 (a-f). One dimensional screening of mineral based media components

maltose produced the highest biomass under the same operating conditions. In the case of nitrogen source, the shake flask experiment showed  $\text{NH}_4\text{Cl}$  as the most favored nitrogen source for the same. The results are summarized in Fig 2 and Fig 3.

#### Optimization of mineral based medium ingredients

One factor at a time experiment shows the optimum of the various components in mineral based medium, such as maltose 2%,  $\text{NH}_4\text{Cl}$  0.1%,  $\text{KH}_2\text{PO}_4$  0.5%,  $\text{MgSO}_4$  0.06%,  $\text{CaCl}_2$  0.015% and yeast extract 0.03% (Fig 4 a-f). Absorbance of 1 of yeast cells in suspension (wet weight) corresponds to 0.4669g/L dry weight.

## DISCUSSION

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 -100g/L concentration of NaCl in shake flask experiments. The growth exponentially increased with the concentration of NaCl from 0 - 40g/L but the production slightly decreased at concentrations 50-80g/L NaCl and noticeably decreased from 80-100g/L. Maximum biomass was obtained at the concentration of 20g/L. 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. Yeast requires carbon and nitrogen sources and also a range of metals such as magnesium, sodium, potassium, iron, zinc, copper and manganese and other inorganic nutrients such as chloride, sulphur and phosphate. The present study using shake flask experiments enabled to find the suitable carbon and nitrogen sources for the maximal production of *Candida* MCCF 101. For determining the effects of carbon and nitrogen sources on the biomass production, cultivations were performed in mineral based medium with 1g/L of different carbon source and 1g/L of nitrogen sources. Among the various sources studied, the highest biomass yield of *Candida* MCCF 101 was obtained on incorporating maltose as single carbon source and  $\text{NH}_4\text{Cl}$  as the nitrogen source. Glucose, sucrose, starch, xylose and lactose were found to be poor carbon sources for *Candida* MCCF 101. Accordingly, maltose and  $\text{NH}_4\text{Cl}$  were chosen as the carbon and nitrogen sources for further experiments.

One dimensional screening (initial screening experiment) of different media components was carried out in order to find the significant ranges of medium components affecting the biomass production. From this method the individual effects of medium components could be seen on a graph without the need to revert to more sophisticated statistical analyses. The experimental data revealed that the *Candida*

MCCF 101 had significant biomass production at optimum of maltose 2%,  $\text{NH}_4\text{Cl}$  0.1%,  $\text{KH}_2\text{PO}_4$  0.5%,  $\text{MgSO}_4$  0.06%,  $\text{CaCl}_2$  0.015% and yeast extract 0.03%.

## CONCLUSIONS

Over the years marine yeasts have been gaining increased attention in animal feed industry due to their nutritional value and immune boosting property. Marine yeasts are regarded as a safe feed material easily amenable to biotechnological processes. Aquaculture field still lacks a feed composed of single cell protein (SCP) from marine yeast with high content of protein and other nutrients. The marine yeast *Candida* MCCF 101 can be mass produced in a medium composed of maltose 2%,  $\text{NH}_4\text{Cl}$  0.1%,  $\text{KH}_2\text{PO}_4$  0.5%,  $\text{MgSO}_4$  0.06%,  $\text{CaCl}_2$  0.015%, yeast extract 0.03%,  $\text{NaCl}$   $20\text{gL}^{-1}$ , at pH 4.5 and temperature  $25^\circ\text{C}$ .

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