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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating 2017: 5.03
TPI 2017; 6(8): 22-25

www.thepharmajournal.com
Received: 16-06-2017
Accepted: 17-07-2017

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Optimization of low-cost substrate for the production of single cell protein using *Kluyveromyces marxianus*

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Abstract
Advent of 21st century has brought an exponential growth in population thereby generating a great threat to food availability and agriculture especially in less developed countries. Single cell protein (SCP) production at a large scale is believed to be a pragmatic solution to the problem of global food shortage. The present work aims at optimizing the growth conditions and identifing a low cost substrate for the production of SCP using *Kluyveromyces marxianus. K. marxianus* is known to have a wide range of application in food industries and in the field of biotechnology. The parameters selected to test the optimum growth conditions were pH, temperature, varied combination of carbon and nitrogen sources. It was found that the strain of *K. marxianus* recorded maximum growth at pH 7 and temperature of 30°C. Three carbon sources i.e. lactose (4%), maltose (4%) and glucose (4%) and three nitrogen sources i.e. ammonium nitrate (1%), di-ammonium hydrogen phosphate (1%) and yeast (0.5%) + peptone (0.5%) were tested in all possible combinations and the maximum growth results was obtained with the combination of lactose and yeast extract + peptone. Whey was identified as a cost effective raw material for the production of SCP and uninterrupted fermentation was carried out under laboratory conditions wherein 80% of biomass and protein content was harvested within 24 h. At this time the biomass yield was 36 mg/ml in which the crude protein content was 83.33%.

Keywords: SCP, Whey, *Kluyveromyces marxianus*, protein, fermentation

1. Introduction
With an exponential growth in human population, world is facing the problem of protein shortage. Single cell protein (SCP) production can be a promising alternative wherein dead; dried cells of microbes viz. yeast, fungi, bacteria and algae can be used not only as feed but also as a valuable addition in human diet to enhance its nutritive value. This may help to alleviate the condition of food shortage especially in the less developed countries where the population growth is much higher (Tannenbaum and Wang, 1975) [1]. Production of SCP has contributed to value addition by conversion of wastes or by-products into cost effective commercial products with high nutritional profile. Large scale production of SCP is not only a remunerative venture but can also be used for eradication of malnutrition in developing countries. (Ugalde and Castrillo, 2008) [8].

High multiplication rate of microbes; cost effectiveness; ability to grow on a variety of low cost carbon and nitrogen sources and high content of protein (around 70 to 80%) from microorganisms; production of microbes is independent of seasonal factors (Lee, 1996; Waites *et al*, 2001) [10, 21] are the various factors makes SCP from microorganism a better alternative of protein for food or feed as compared to plants and animals. However, the limited usage of SCP by humans is related to its high content of nucleic acids. The nucleic acid content can be markedly reduced using alkaline or acidic hydrolysis and activation of endogenous RNA-ases (Lee, 1996) [10] thereby utilizing the SCP to its maximum potential.

Microbial biomass finds a broad range of application in food industry such as starter cultures for food and beverage fermentations, waste treatment processes and agricultural inoculants, animal feed, protein sources for humans as well as functional foods for the treatment of various chronic diseases (Lee, 1996; Waites *et al*, 2001) [10, 21]. Strains of *Kluyveromyces marxianus* are found in diverse habitats, which resulting in a wide range of metabolic diversity and a considerable degree of intraspecific polymorphism. This led to its varied applications in the field of biotechnology and food science which is consistently investigated over the period of years. The objective of the study was to identify the suitable substrate for the biomass production using *Kluyveromyces marxianus* under controlled fermentation which can be utilized for large scale industrial production of SCP.

2. Materials and Methods

2.1. Sampling and Isolation of Yeast Strains

Milk samples from a local dairy in Lucknow, India were collected in sterile 500 ml bottles and were brought to the laboratory in an ice box within 2 h. Inoculation of yeast was done by adding 10 ml of the milk sample to 90 ml of yeast extract glucose chloramphenicol agar (YGCA) medium. 0.1 g/l chloramphenicol was used to cease any becterial growth and pH was maintained at 4.5. The incubation with constant shaking at 120 rpm was allowed at a temperature of 28 °C for 24h.

The yeast cells in media were studied by optical microscopy. The yeast strains were isolated on spread plates made from serial dilutions and incubation temperature of 28 °C was maintained for 72 h. Colonies with distinct morphological differences were selected and purified by streaking on potato-dextrose agar (PDA) (Bainotti *et al*, 1987; Omer and Sabry, 1991; Bury *et al*, 2001) [1, 14, 3]. Identification of the yeast strain was first done by microscopic examination.

2.2. Optimization for growth

The growth of potential strain was optimized varing environmental parameters viz. pH, temperature and different combinations of carbon and nitrogen sources on the growth of the potential strain. The optimum pH for the growth of isolated strain was tested between the range of 4 to 9. The culture was inoculated in different test tubes and was left for incubation in a rotary shaker at 120 rpm. The absorbance was measured at 620 nm at different intervals. Similarly, growth at varied temperatures viz. 25 °C, 30 °C, 35 °C and 40 °C was analyzed. The incubation period was varied from 0 to 60 hrs was studied. Different carbon sources namely glucose, maltose, and lactose and nitrogen sources namely diammonium hydrogen sulphate, ammonium sulphate and yeast extract + peptone were added at 4% and 1% respectively in various combinations. Growth was measured at 620 nm at different time intervals.

2.4. Identification of suitable substrate

Under the varied optimized conditions, the substrate for SCP production was explored and the one having high potential favoring the growth of *Kluyveromyces marxianus,* was selected for the biomass production.

2.5. Production of Biomass and Protein estimation

The substrate was adjusted to optimum pH by addition of di-potassium hydrogen phosphate (basic) and potassium di-hydrogen phosphate (acidic) and was sterilized by boiling at 100 ºC for 15 min. To prevent protein precipitation during sterilization, the proteins were eliminated from the substrate by cooling and filtering the protein sediments. 1 litre of the filtrate obtained was sterilized at 115 ºC for 10 min and then inoculated with 5% of actively growing isolates. The medium was incubated at 28 ºC for 5 days with constant shaking at 150 rpm (Lenore, 1989). After incubation, the biomass of the yeast strain was obtained by centrifugation at 4000 rpm and washed twice with distilled water. Dry weight of biomass was determined after drying overnight at 105 ºC. The protein percentage of biomass was estimated by micro-kjeldahl method with 6.25 as conversion factor.

3. Results and Discussions

3.1. Isolation and Identification of yeast strains

After phenotypic characterization using microscopic examination, the strain of *Kluyveromyces marxianus* was identified based on colony colour, shape, texture and morphology.

3.2. Optimum condition for the growth of *Kluyveromyces marxianus*

The maximum growth of *K. marxianus* for biomass production was achieved at pH 7. The growth rate of *K. marxianus* at various pH ranging from 4 to 9 has been represented in Fig. 1. Similar results was found by Kundu *et al*, 2012 [9] where it was reported that the optimum pH for the growth of *Kluyveromyces marxianus* MTCC 4059 is 7. It was also reported that maximum growth was observed at a temperature of 30 ºC (Kundu *et al*, 2012) [9] which is also in accordance with the results whereby four different temperatures of 25 °C, 30 °C, 35 °C and 40 °C were tested and the maximum growth was seen at 30 °C (Fig. 2). Growth analysis of nine different combinations of carbon and nitrogen represented in Fig. 3 revealed that the combination of Lactose (4%) and yeast extract (0.5%) + peptone (0.5%) was most suitable for the growth of *K. marxianus.* Similar results pertaining to the growth conditions of *K. marxianus* have been reported by Fonseca *et al*, 2013 [5] and Oliveira *et al*, 2012 [13].

3.3. Selection of low cost substrate and SCP production

K. marxianus releases enzymes to modify whey for the production of single cell protein (Chandrani-Wijeyaratne and Tayathilake, 2000) [4]. The results clearly indicates that growth of *K. marxianus* was maximum in lactose containing substrate (Fig. 3). Several studies have also reported earlier that whey is an excellent carbon source for SCP production due to its high lactose content (Somaye *et al*, 2008) [19]. In solute form it is 5 to 8% while in dried form it is around 70% (Speer, 1998; Somaye *et al*, 2008) [20, 19]. Rizvi and Josephson, 1975 reported that whey has a substantial amount of non-protein nitrogen content. Unlike other dairy product like curd, yoghurt, cheese, etc, whey possess greater liquidity at room temperature enabling improved fermentation process. Whey being a by-product of dairy industry, it is extremely cheap and easily available. Improper disposal of whey is often associated with serious environmental problems (Becerra *et al* 2004; Ghaly *et al*, 2004; Kotoupas *et al*, 2007; Rubio-Texeira *et al*, 2000; Waites *et al,* 2001) [2, 6, 7, 17, 21].

In line with the ongoing discussion, whey is the most suitable substrate for a large scale profitable production of SCP. Successful production of SCP was performed at a laboratory scale and it was found that a characteristic growth curve was obtained with whey as a substrate. Fig. 4 shows the biomass production and protein content with respect to time. It was observed that after 24 hrs of uninterrupted process around 80% of total protein can be harvested. At this time the biomass yield was 36 mg/ml in which the crude protein content was 83.33%.

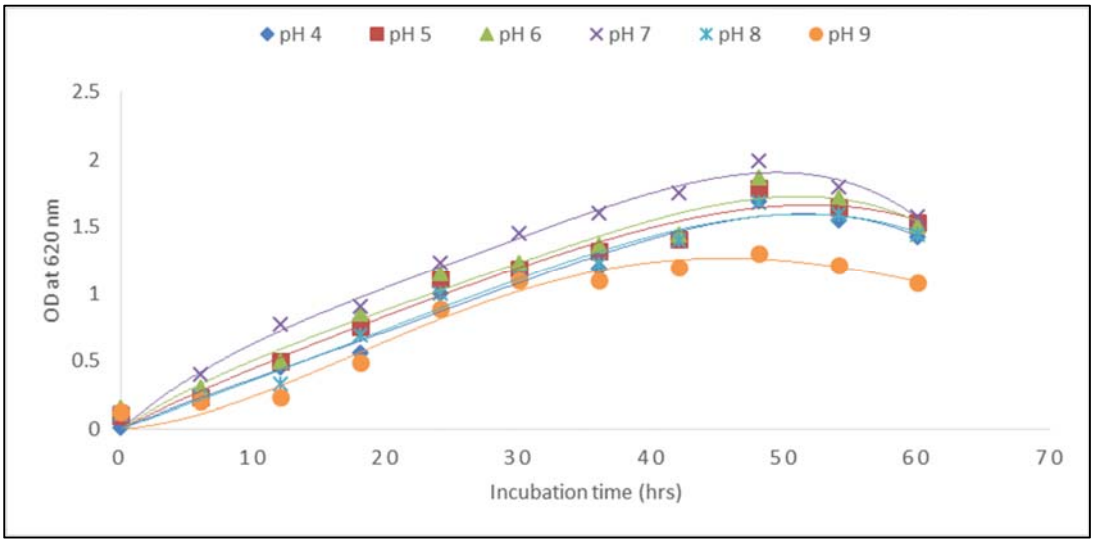

Fig. 1: Effect of pH on the growth of *K. marxianus*

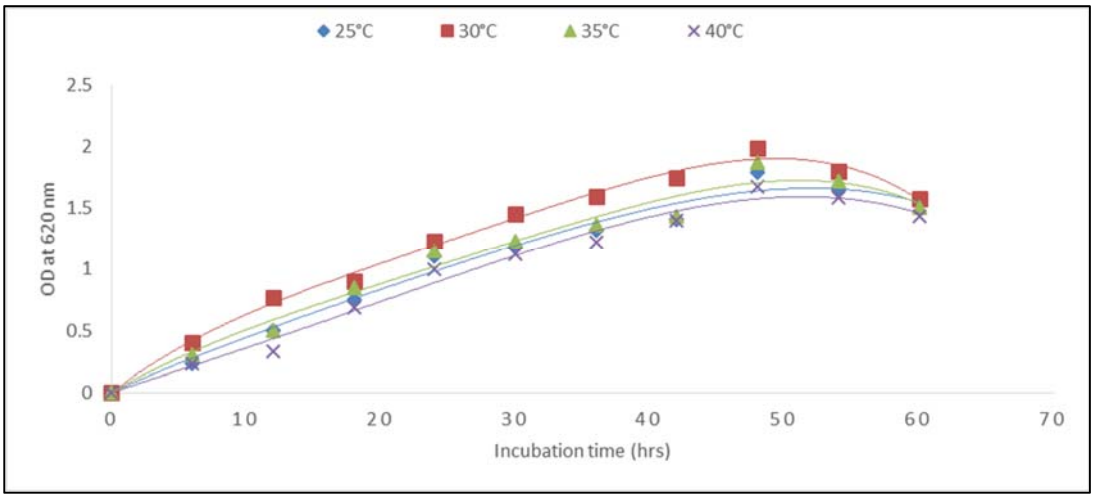

Fig. 2: Effect of temperature on the growth of *K. marxianus*

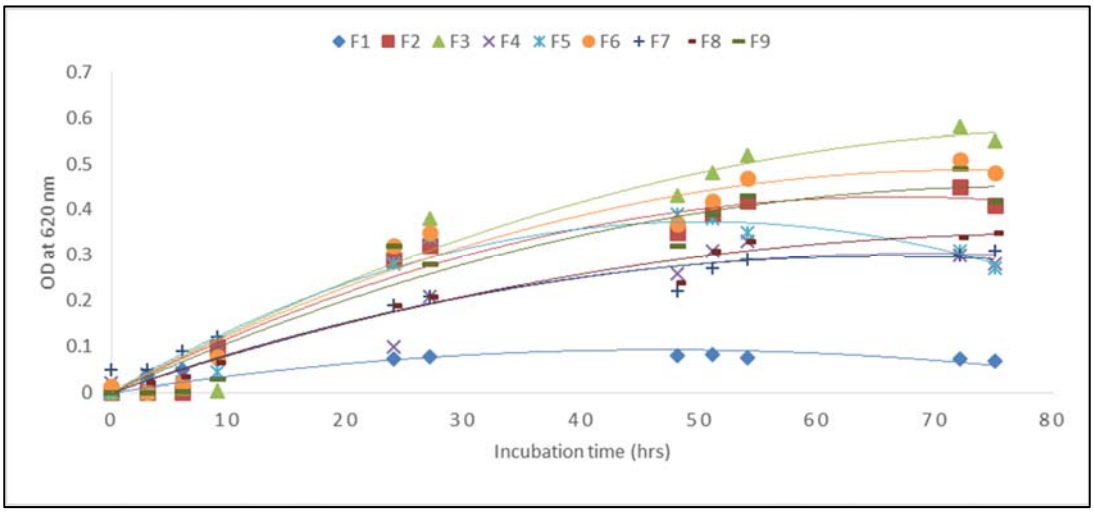

Fig 3: Effect of various combinations of carbon and nitrogen sources on the growth of *K. marxianus*

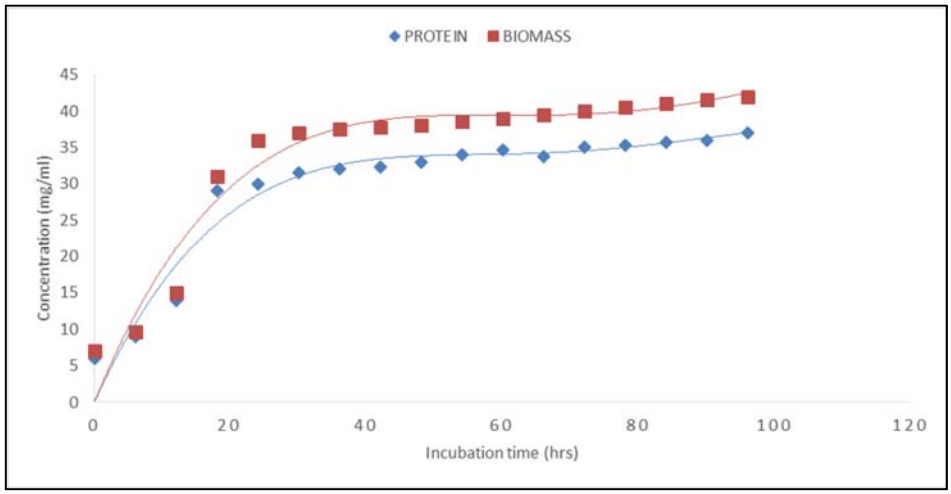

Fig. 4: Effect of incubation period on Biomass and Protein concentration of *Kluyveromyces marxianus*

4. Conclusion

Results of the study indicated that whey is a suitable substrate for the production of Single Cell Protein using strains of *Kluyveromyces marxianus.* A profitable yield can be obtained within 24 h of fermentation process with high protein content. At this time the biomass yield was 36 mg/ml in which the crude protein content was 83.33%. The nucleic acid content must be reduced to permitted level prior to further use of produced SCP. Recently novel methods for nucleic acid reduction have been worked on; however, the same may be carried for further studies.

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