BIOTECHNOLOGICAL UTILIZATION OF DAIRY WASTE TO SOLVE ENVIRONMENTAL PROBLEM

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ABSTRACT

To find an alternative mold source, nine molds were cultured in lactase production medium at room temperature in static condition for 7 days.Experimental results showed that *Aspergillus* species (L_9) has maximum lactase activity at temperature 30° C after 5 days incubation. The optimized pH, size of inoculum and age of inoculum for maximum lactase production were 5.0, 6% v/v and 7 days. Lactase yield was determined by inoculating deproteinized cheese whey by hyperproducer under optimized conditions of fermentation.The best conditions for optimum activity of crude enzyme were determined. Lactase activity was maximum at pH 6.0 and temperature 45°C. The enzyme was stable upto 10 minutes at pH 6.0 and temperature 45°C. It was concluded that *Aspergillus* species (L_9) could be used as an alternative for production of lactase on industrial scale utilizing deproteinized cheese whey. This strain can be used for removal of whey pollutants, SCP and ethanol production and treatment of lactose in dairy food.

Key Words: Lactase, *Aspergillus*, Deproteinized whey, Lactose intolerance Mold

INTRODUCTION

Commercial lactase is produced from both yeasts , such as *Kluyvermyces lactis* and *Kluyvermyces fragilis* and molds, such as *Aspergillus niger* and *Aspergillus oryzae*[1]. Whey is the aqueous fraction of milk generated as a by-product of cheese manufacturing which is produced in large amounts .The main solute in cheese whey is lactose, present at a concentration of about 4.5-5%.Other components are proteins, salts and vitamins that are present in minor amounts. The low concentration of these components makes their recovery uneconomical .Because of its high organic content, dumping directly to the environment causes contamination problems. As a solution, bioconversion of whey into lactase has been performed in several countries[2]. Lactose content of whey reaches 4.8%,and it includes relatively high levels of other nutrients that make it suitable as a microbial culture medium .Microorganisms capable of using lactose as the sole carbon and energy source are producers of beta-D-galactosidase, an enzyme that breaks down lactose to glucose and galactose[3]. The selection of an inexpensive and easily available substrate together with a suitable producer microorganism, optimization of culture conditions, and effective downstream processing are essential to reduce the cost of enzyme preparation[4].

Treatment of milk and milk products with lactase (β-D-galactosidase, EC 3.2.1.23) to reduce their lactose content seems to be an appropriate method to increase their potential uses and to deal with the problems of lactose insolubility and lack of sweetness. Furthermore this treatment could make milk, a most valuable food, available to a large number of adults and children intolerant to lactose[5].

AIMS AND OBJECTIVES

The present study deals with the determination of growth conditions for maximum lactase

production by hyperproducer mold (*Aspergillus* species -L_9) grown in cheese whey. Some properties of crude lactase were also determined.

MATERIAL AND METHODS

Microbiological media and chemicals

Ingredients of bacteriological culture media and chemicals were obtained from Hi- Media Private Limited, Bombay. All the chemicals used were of analytical grade. Whey was brought from local dairies.

Methods

Sample collection

For isolation of molds, soil samples were collected from vicinity of local dairies. All samples were collected in sterile polythene bags.

Isolation of molds

Potato Dextrose Agar (PDA) was used for isolation of the molds. For isolation of molds, the standard pour plate isolation technique was used. 1 g of soil sample was suspended in 10 ml distilled water. This soil suspension was diluted as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-5} using sterilized distilled water. 0.1 ml of most diluted suspension was transferred aseptically over a layer of PDA. The plates were incubated at room temperature for 7 days. The isolated colonies were grown on PDA slants by incubation at room temp for 5 days[6].

Maintenance of mold cultures

Stock cultures were produced on PDA in five days and maintained at 4^0C[6].

Primary screening

The medium described by Fiedurek and Ilczuk was used for screening[7]. The lactase producing potential of mold isolates was determined as follows.The cultivation of mold isolates was performed in 250 ml conical flask with 25 ml of Fiedurek and Ilczuk medium in stationary condition at room temperature. 5% v/v dispersion of mold spores washed out from PDA slants by scratching using 10 ml sterile distilled water was used to inoculate 25 ml sterilized medium. The inoculated flasks were incubated for 7 days at room temperature. Culture broth was separated from mycelium by centrifugation at 7200 rpm for 10 min and the supernatant was used as crude enzyme.

Determination of lactase activity (Lactase assay)

Lactase activity was determined by incubating 1 ml of substrate solution (2.5 milligrams lactose dissolved in 0.1M sodium acetate buffer , pH 5) with 1 ml crude enzyme at 50^oC for 5 minutes. Reaction was terminated by adding 1 ml of 10% sodium carbonate in the reaction tube. The absorbance was read at 420 nm using UV-VIS spectrophotometer. The amount of reducing sugar produced was determined with DNS methods. 1 unit of lactase activity was described as the amount of enzyme producing 1µ mole of glucose in 1 ml medium at 50^oC in 1 min[1].

Identification of mold isolate

The isolated mold strain, which was found most efficient in lactase production, was identified by standard procedure[8,9].

Fermentation studies

Inoculum preparation

A pure efficient hyper producer (mold isolates L_9) isolated from soil was used throughout this study. The inoculum in this study was prepared by inoculating a loopful of culture from 5 days old PDA slant in to PD broth (10 ml). The medium was incubated at room temp for 5 days, 5% (v/v) inoculum was used for further studies.

Fermentation system

Cultivation of hyperproducer was done in 250 ml Erlenmeyer's flask containing production medium with composition (g/l): 10 gm lactose, 1.5 g peptone,1.0 g yeast extract,1.0 g potassium Dihydrogen phosphate,1.0 g magnesium sulphate,7.0g ammonium dihydrogen phosphate,0.3 g calcium chloride under static condition[7].

Determination of optimum conditions for lactase production

The optimization of cultural conditions was carried out based on stepwise modification of governing parameters for lactase production. Each optimized parameter was employed in subsequent experiments. The effect of different physical parameters like incubation period (3-11 days), Inoculum densities (2-8% v/v), age of inoculum (3-11 days) and incubation temperature (25-35^oC) were studied on production of lactase by selected *Aspergillus* sp.-L_9

Determination of general properties of crude extracellular lactase

Production of lactase using whey medium (the cheese whey supplemented with other nutrients of fermentation medium) under optimum conditions. Whey was obtained from a local dairy. After adjusting the pH to 5 with 5N HCl, whey was heated at 121^{o}C for 15 min to denature the proteins and the precipitate were removed by centrifugation at 10,000 g for 15 min. The supernatants were adjusted to pH 5, sterilized at 121^{o}C for 15 min[9].The crude enzyme used in study of enzyme kinetics was obtained by growing hyperproducer (*Aspergillus* species) in cheese whey medium supplemented with nutrients of fermentation medium except lactose before sterilization[7,9]. The sterilized medium was inoculated with 6% v/v inoculum. The inoculated medium was incubated at 30^{o}C for 5 days in stationary condition. After fermentation the fermented medium was filtered and the filtrate was used as a crude enzyme.

Determination of optimum pH

Optimum pH for maximum lactase activity was determined by assaying lactase over pH range 3-8 using 0.1 M sodium acetate buffer at 50^{0}C for 5 mins.

Determination of optimum Temperature

Optimum temperature for maximum lactase activity was determined by assaying lactase over the temperature range 25-75^{o}C in 0.1 M sodium acetate buffer (pH 6.0) for 5 mins.

Enzyme stability at optimum pH and Temperature

The stability of lactase was determined at 45^{o}C with reaction medium containing 1ml of enzyme preparation in 0.1 M sodium acetate buffer (pH 6.0) for 5-30 mins.

RESULTS AND DISCUSSION

The production of enzyme by microorganism is great achievement in the field of fermentation technology. It is evident that enzyme production strongly dependant on selection of strain. Molds are one of the important organisms having wide industrial applications. Most of the molds are unable to assimilate lactose as carbon source. Mold that produce extracellular lactase efficiently was determined in this study.

Isolation of mold isolates

Nine soil samples were collected from vicinity of dairies of the city. In this study, 9 molds were isolated by using PDA by serial dilution technique.

Potential of mold isolates to produce lactase

In our study, 9 mold isolates from various soil samples were tested for the production of lactase. Mold isolate (L_9) was found most efficient in lactase production.

Identification of selected mold isolate

The L_9 mold isolate producing maximum lactase was identified by standard procedure and it was identified as *Aspergillus* species, on the basis of cultural characteristics.

1 Brown colored colonies on PDA after 4 days incubation at 25^{o}C.
2 Vegetative mycelium septate and branched.
3 Conidiophore's unbranched.
4 Conida are globose to subglobose in shape, rough surface texture and produced in chain.

The above characteristics (3 and 4) were studied by observing *Aspergillus* sp. stained with lactophenol cotton blue under 100$_{x}$ magnification of bright field microscopy.

In similar study found in literature, lactase production from *Aspegillus niger, Penicillum* and *Fusarium* sp. were investigated[10].

Optimization of fermentation conditions for lactase production

In this study, the basal medium was optimized in order to improve lactase production.

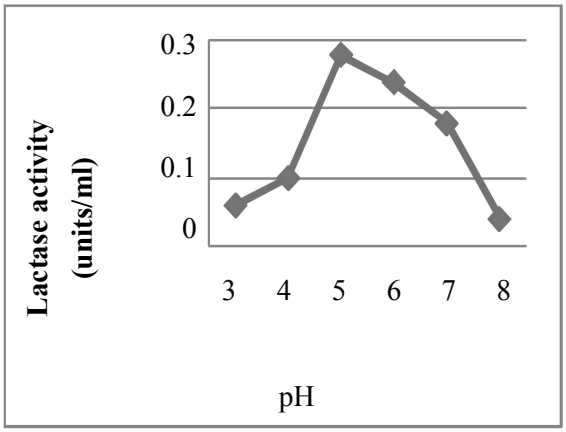

Fig.1 : Effect of pH on lactase production

Effect of initial pH

Maximum production of lactase was observed when the initial pH of the medium was adjusted to 5.0 **(Fig.1).** Low pH of the medium is desirable as it will reduce the possibility of contamination as shown by wendorff et al[5].

Sonia A. et al produced lactase by *Candida pseudotropicalis* grown in deproteinized whey. Maximum enzyme production was obtained in 2% whey medium with optimal initial pH (3.5) for cultivation[11].

Time course study

Influence of incubation time on lactase production was evaluated. Incubation period for maximum lactase production was found to be 5 days, by increasing or decreasing duration of incubation period, lactase production decreases. **(fig.2)**

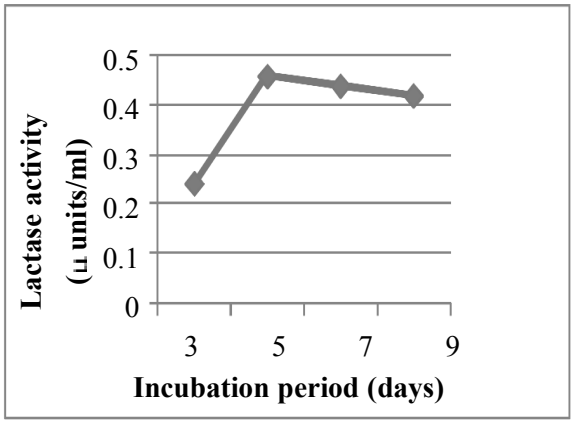

Fig.2 : Time course study on lactase production

Ramana Rao et al. studied beta-galactosidase production by *S. thermophilus* up to a period of 60 h. A progressive increase in enzyme units available was observed upto 24 h of incubation, after which it became constant[12].

Effect of size of inoculum

Inoculum level for optimum production of lactase by *Aspergillus* species. was worked out. Increase in quantity of inoculum increased lactase titre 6% v/v inoculum gave the highest titre in 5 days. **(fig.3).** At low concentration, the number of cells were not well enough to utilize essential amount of substrate to produce enzyme. At high concentration of inoculum, viscosity of the fermentation media increased due to the tremendous growth of mold resulting in nutritional imbalance in medium.

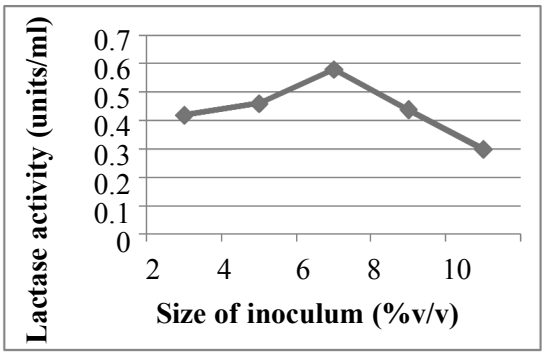

Fig.3 : Effect of size of inoculum on lactase production

Use of stronger inoculums can reduce the lag phase and significantly reduce the fermentation time. However the yeast culture will always need sufficient time for beta-galactosidase secretion and to initiate lactose hydrolysis[4].

Effect of age of inoculum

Age of inoculum had no appreciable effect except that young inoculum gave less lactase **(fig.4).**

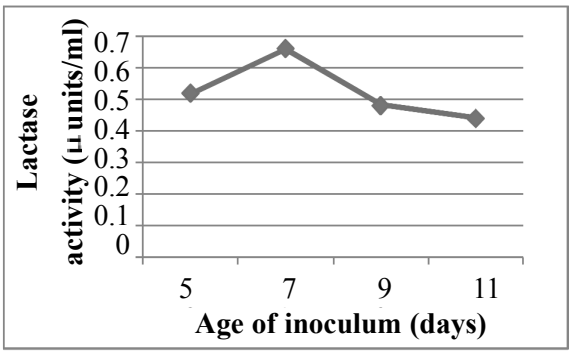

Fig. 4 : Effect of age of inoculum on lactase production

Effect of incubation temperature

Temperature of medium plays an important role in production of lactase. Optimum temperature for maximum lactase production was found to be 30°C (mesophilic). By lowering the temperature; below optimum or increasing the temperature above optimum lactase production decreases. **(fig.5)**

Higher temperature is found to have some effects on metabolic activities of microorganisms and cause inhibition of the growth of the fungus. The

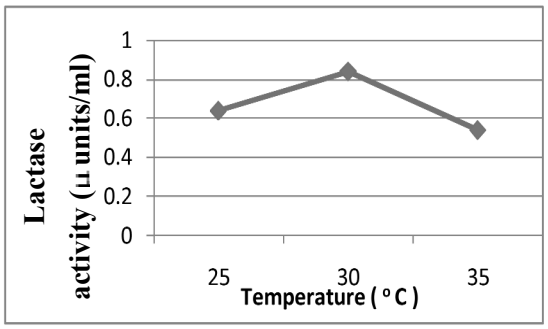

Fig.5: Effect of temperature on lactase production

enzymes for mold growth are denatured by losing its catalytic properties at higher temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure.

Enzyme activity of crude lactase obtained by growing *Aspergillus* species in whey medium

Enzyme activity of crude lactase was 0.92 u/ml.Ramana M.V et al extracted beta-D galactosidase (Ec 3.2.1.23) from *Streptococcus* thermophilus grown in deproteinized cheese whey. Optimum cultural conditions for maximum enzyme production was pH 7.0, 40°C and 24h.[12].

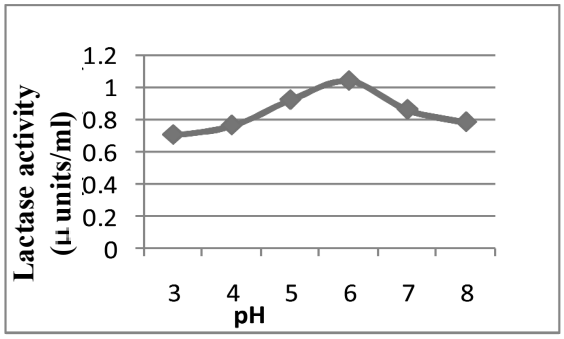

Fig. 6a : Effect of pH on lactase activity

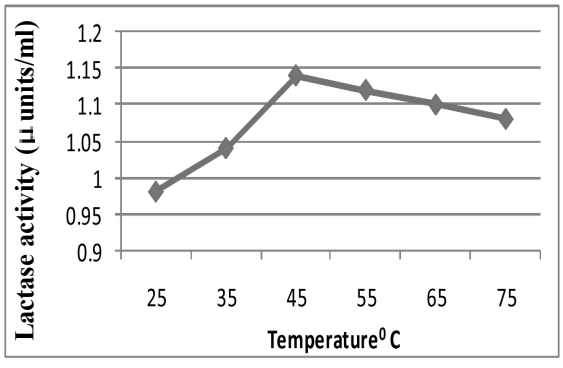

Fig. 6b : Effect of Temperature on lactase activity

Enzyme Characterization

Optimum pH and temperature

Maximum hydrolysis of lactose occurred at pH 6.0 and at temperature 45°C. **(Fig. 6A** and **Fig. 6B)**

pH and thermal stability

Stability is an important criteria for an enzyme intended for use on industrial scale[1]. Therefore in the second part of the study, pH and temperature stability of lactase were investigated for an enzyme.

The enzyme was stable at pH 6.0 and temperature 45°C upto 10 min. **(Fig.7)**. As the enzyme was stable at pH 6, it can be used for treatment of whey (having acidic condition).The enzyme was also used for treatment of milk (pH 7) as it showed activity at neutral pH .

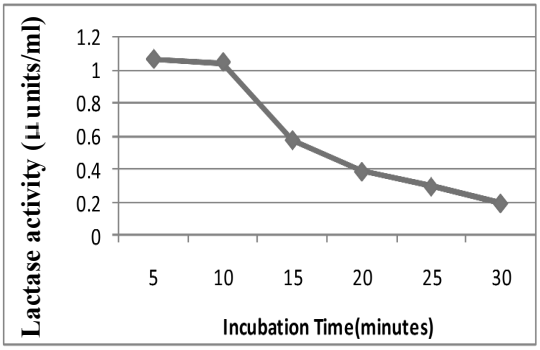

Fig.7 : pH and Temperature stability of lactase

Scyis, I et al reported in his study that lactase of *Trichoderma* species by showed enzyme activity above 90% in the pH range of 3.0 - 7.5, which implies that the produced enzymes can be used both in whey, having acidic pH and in milk, having neutral pH[1]. As the enzyme was stable at 45°C,it can be preferred for industrial processes.

CONCLUSION

It may be concluded that Aspergillus species (L_9) could be the best potential source of lactase. Lactase from this mold isolate can be used for the treatment of lactose intolerance by reducing lactose content of milk.Growing the mold isolate for lactase production in whey can reduce the

lactose content of whey and may help to prevent environmental pollution.

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