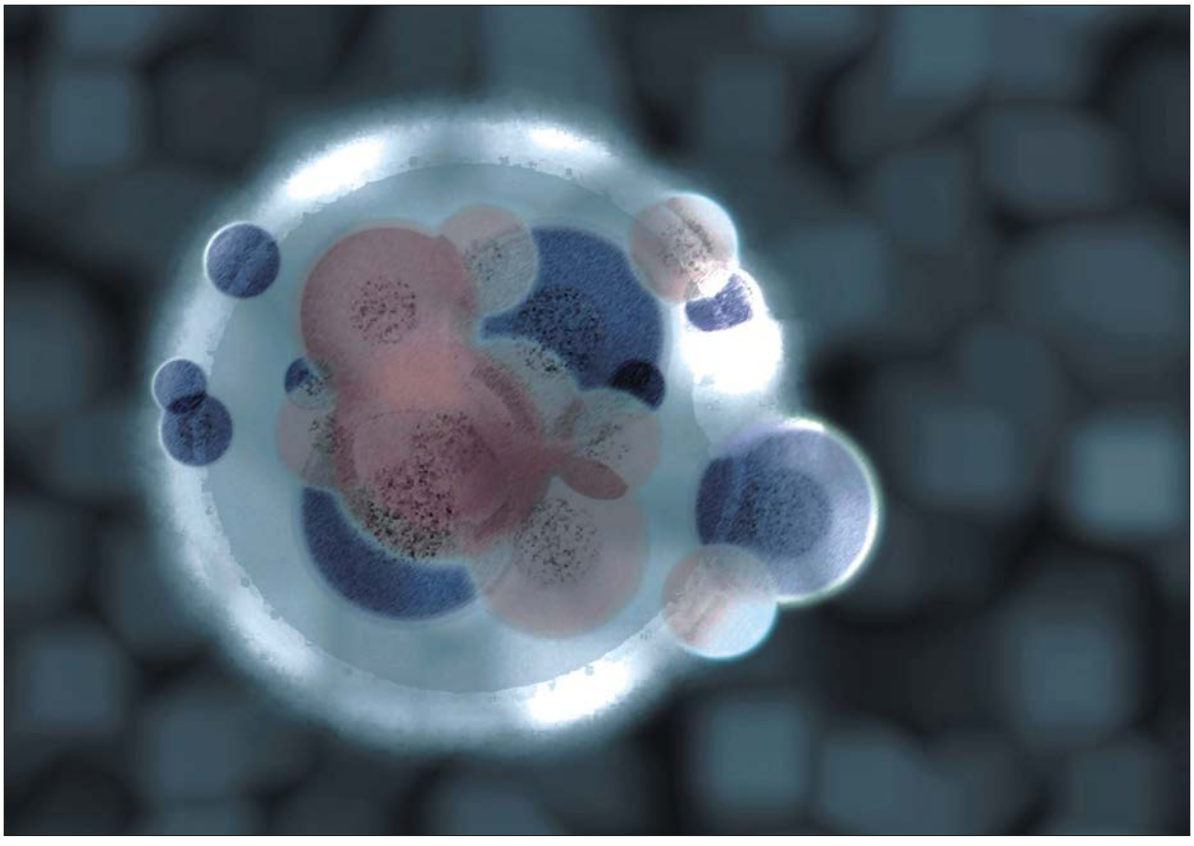

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 6 (1): 193-197, 2006
ISSN 1727-3048

Process Optimization for β-D-Galactosidase Production Using Yeast Culture

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Abstract: The use of *Kluyveromyces marxianus* NCIM 3465 for the production of β-D-galactosidase is reported. Experimentation was carried out to optimize the process parameters for maximum enzyme production by varying the parameters such as pH of the medium, temperature, inoculum size, age of inoculum, agitation and incubation time. The maximum enzyme activity was observed with pH 5.0, temperature 30°C, inoculum size 6% (v/v) having 20 h age, under shaking conditions (100 rpm) after 24 h of incubation.

Key words: Yeast, β-D-galactosidase, enzyme activity

INTRODUCTION

The use of β-D-galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2, 1.23), most commonly known as lactase, for the production of lactose hydrolyzed milk and whey hydrolysis to obtain glucose and galactose constitutes one of the most important applications of biotechnological processes[1]. This enzyme has also been suggested to prevent lactose crystallization in dairy foods by using lactose hydrolysed milk. The lactose hydrolyzed milk may be consumed directly or may be used to produce many products such as dehydrated products, concentrates, cheeses, yoghurts, buttermilk especially suitable for the lactose intolerants. Lactose hydrolysis causes several changes of potential values on manufacture and marketing of dairy products including increased solubility, sweetness, broader fermentation possibilities, more ready fermentation of carbohydrates and reduced lactose concentration with associated diminished possibility of lactose crystallization[2]. The lactose hydrolysis in whey can help to solve the pollution problem caused by whey and whey permeates. High concentration of lactose in whey is a major environmental problem since its disposal in local water streams increases the biological oxygen demand manifolds[3].

β-D-Galactosidase occurs widely in nature and is produced by a number of microorganisms, plants and animals. However, their properties differ markedly according to their source and microorganisms offer high yields as compared to other sources. The yeast *Kluyveromyces* has been reported to be the most important source for the production of β-D-galactosidase as the enzyme from the yeast has an optimum pH suitable for lactose hydrolysis in milk[4,5]. The application of β-D-galactosidase technology for production of lactose hydrolyzed milk for lactose intolerant persons and whey hydrolysis to obtain glucose and galactose have several applications in the food, dairy and fermentation industries[1]. Moreover, this enzyme has also been applied in the synthesis of oligosaccharides, which are increasingly being recognized as useful dietary tools for the modulation of the colonic microflora toward a healthy balance[6]. Because of the technological importance of this enzyme, β-D-galactosidase has attracted keen interest by researchers during the last decade. In view of the above, the present study was carried out to optimise the different process parameters for β-D-galactosidase production using a yeast strain.

MATERIALS AND METHODS

Microorganism: *Kluyveromyces marxianus* NCIM 3465 was procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune (India).

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Maintenance of the culture: The culture was revived on maintenance medium containing (w/v) malt extract (0.3%), yeast extract (0.3%), peptone (0.5%) and glucose (1.0%). The culture was incubated at 30°C for 48 h and maintained for fortnightly intervals on agar slants at 4°C.

Preparation of starter culture: The maintenance media (50 mL lots) were taken in Erlenmeyer flask (250 mL), sterilized by autoclaving at 15 psi for 20 min, cooled and inoculated with a loopful of culture from slant and incubated at 30°C for 24 h.

Production of β-D-galactosidase: The fermentation media used for the production of enzyme, as optimised in lab was composed of lactose (5%), peptone (0.5%), yeast extract (0.3%), ammonium sulphate (0.2%) and potassium dihydrogen orthophosphate (0.1%). The 50 mL fermentation media contained in 250 mL flask were inoculated with 24 h old inoculum (unless otherwise mentioned), incubated at specified times under shaking conditions. After specific time intervals, the samples were drawn from flasks and assayed for enzyme activity.

Enzyme assay: The assay for measurement of enzyme activity was followed as per the method of Miller[7]. One unit of enzyme activity is defined as one micromole (μM) of 2-nitrophenol liberated per min under standard assay conditions.

Optimization of process parameters: The various process parameters such as pH, temperature, age of inoculum, inoculum size, agitation and incubation time were optimized by varying the respective parameters.

RESULTS AND DISCUSSION

The effect of following process parameters was monitored to enhance the β-D-galactosidase activity during the course of the present investigation.

Effect of pH: The hydrogen ion concentration of an environment has the maximum influence on the microbial growth. The pH affects at least two aspects of microbial cells i.e. functioning of its enzymes and the transport of nutrients into the cell. It limits the synthesis of metabolic enzymes responsible for the synthesis of new protoplasm. The pH values also affect the RNA and protein synthesis[8]. When microorgamisms are grown on either side of their optimum pH range, there may be an increased lag phase. The pH 5.0 has been used for β-D-galactosidase production using different yeast strains[9]. However, a pH 5.5 has been used for the enzyme production using *K. marxianus*[10], whereas, a pH 4.68 has been found optimum for β-D-galactosidase production using *K. lactis*[11].

Table 1: β-D-galactosidase production by *K. marxianus* NCIM 3465 with pH as a function

pH	Enzyme activity (IU mg^{-1} dry weight)
4.0	1.46
4.5	1.53
5.0	1.56
5.5	1.55
6.0	1.52

Table 2: β-D-galactosidase production by *K. marxianus* NCIM 3465 with temperature as a function.

Temperature (°C)	Enzyme activity (IU mg^{-1} dry weight)
20	1.42
25	1.55
30	1.56
35	1.44
40	0.19

The effect of hydrogen ion concentration on β-D-galactosidase production was evaluated by using fermentation medium having a pH range of 4.0-6.0. The results of the enzyme activities (Table 1) showed maximum activity of 1.56 IU mg^{-1} dry weight at pH 5.0. At higher and lower pH levels, a decrease in the enzyme activity was observed.

From the present observations, pH 5.0 was considered optimal for maximum β-D-galactosidase activity. In the subsequent experiments, the pH of the fermentation medium was adjusted to 5.0.

Effect of temperature: The temperature is also one of the important factors, which influences the activity of metabolic enzymes. Enzymes are most active at optimum temperature and enzymatic reaction proceed at maximum rate. However, below and above optimal temperature reaction rate is decreased which causes the problems in cell metabolism. Temperatures in the range 28-30°C have been used by many researchers[10,12,13] for the production of β-D-galactosidases. Ramirez-Matheus and Rivas[11] have reported 30.3°C as an optimal temperature for β-D-galactosidase production by *K. lactis*.

To find the optimum temperature for β-D-galactosidase production, fermentation medium, after inoculation, was incubated at temperatures in the range of 20-40°C and the enzyme activities were assayed. The enzyme activity increased with increase in the temperature upto 30°C, however, a constant decrease in the activity was found at higher temperature (Table 2). Moreover, a sharp decrease was observed at temperature range of 35-40°C. The maximum enzyme activity of 1.56 IU mg^{-1} dry weight was observed at 30°C.

Table 3: β-D-galactosidase production by *K. marxianus* NCIM 3465 with inoculum age as a function

Inoculum age (h)	Enzyme activity (IU mg^{-1} dry weight)
12	1.37
16	1.51
20	1.60
24	1.56
28	1.55

Table 4: β-D-galactosidase production by *K. marxianus* NCIM 3465 with inoculum size as a function

Inoculum size (%, v/v)	Enzyme activity (IU mg^{-1} dry weight)
4	1.45
6	1.61
8	1.61
10	1.60
12	1.57

From these observations, a temperature of 30°C was considered optimal for achieving maximum enzyme activity and it was adopted for further experimentation.

Effect of inoculum age: To find the effect of inoculum age on β-D-galactosidase production, fermentation medium was inoculated with 12-28 h old cultures. The results (Table 3) showed progressive increase in the enzyme activity, when yeast culture of 12-20 h old was used. The maximum enzyme activity of 1.60 IU mg^{-1} dry weight was observed with 20 h old yeast culture. However, suppression in enzyme activity was observed when 24 and 28 h old growth was used. The low enzyme activity with inoculum age of 12 h may be attributed to the fact that yeast culture may have not yet entered the log phase of growth. The maximum enzyme activity (1.60 IU mg^{-1} dry weight) was observed with inoculum of 20 h, which may be due to the exponential phase of the yeast culture used as an inoculum. Similarly, 20 h old culture of *K. fragilis* for β-D-galactosidase production has been used[14]. However, Ku and Hang[13] have used 24 h old culture of *K. marxianus* strains for β-D-galactosidase production.

Since, 20 h yeast culture displayed maximum β-D-galactosidase activity, it was selected for further studies.

Effect of inoculum size: To study the influence of inoculum size on the β-D-galactosidase activity, different inoculum levels (4-12%, v/v) were added to the fermentation medium (Table 4). β-D-galactosidase activity increased with the increase in inoculum size upto 6% (v/v), thereafter no improvement in enzyme activity was observed. The maximum β-D-galactosidase activity of 1.61 IU mg^{-1} dry weight was observed with 6-8% (v/v) inoculum of yeast culture. The low enzyme activity at 4% (v/v) inoculum level may be attributed to the low density of starter culture. Inchaurrondo *et al.*[9] have used 10% (v/v) inoculum for the enzyme production, whereas, 5% (v/v) has been used for *K. fragilis*[14].

Therefore, an inoculum of 6-8% (v/v) can be considered optimal for achieving maximum enzyme activity using 20 h old yeast culture, however, an inoculum of 6% (v/v) was used in the subsequent studies.

Table 5: β-D-galactosidase production by *K. marxianus* NCIM 3465 with agitation as a function

Agitation (rpm)	Enzyme activity (IU mg^{-1} dry weight)
0	1.37
60	1.50
80	1.58
100	1.61
120	1.60
140	1.59

Effect of agitation: To study the effect of agitation on the β-D-galactosidase production by the yeast culture, the cultivation was carried under stationary condition (control) in a BOD incubator and shaking condition (60-140 rpm) on a rotary shaker (Table 5). The agitation mode of cultivation supported an increase in enzyme activity as compared to the culture maintained under stationary conditions. During the experimentation, a progressive increase in the enzyme activity with increase in the agitation rate upto 100 rpm was observed. However, no improvement in the enzyme activity was observed at higher agitation rates. The agitation speed of 100 rpm displayed maximum enzyme activity of 1.61 IU mg^{-1} dry weight. The increase in enzyme activity with agitation mode may be attributed to the uniform distribution of the yeast culture in the medium resulting in better nutrient availability and oxygen transfer rate. The earlier studies have also supported the agitation mode for β-D-galactosidase production. Champluvier *et al.*[15] have reported the use of 100 rpm agitation for the enzyme production by *Kluyveromyces* sp. The agitation rates of 120 and 130 rpm have been used for the production of this enzyme by other researchers[14,16].

Since, maximum β-D-galactosidase activity was observed with agitation rate of 100 rpm, it was selected for further investigations.

Effect of incubation period: To find out the optimal incubation time for the maximal β-D-galactosidase activity, the fermentation medium inoculated with yeast cultures was incubated for 36 h under the above optimized conditions, samples being drawn at 4 h time intervals from 20 h of incubation (Table 6). An increase in enzyme activity was found upto 24 h and thereafter no

Table 6: β-D-galactosidase production by *K. marxianus* NCIM 3465 with incubation period as a function

Incubation period (h)	Enzyme activity (IU mg^{-1} dry weight)
20	1.58
24	1.65
28	1.64
32	1.61
36	1.59

improvement in this function was observed. However, a decrease in the enzyme activity was observed with further increase in the incubation time (28-36 h). This may be attributed to the growth of the culture having reached the stationary phase and as a consequence of metabolism, microorganisms continuously change the characteristics of the medium and the environment. The maximum production of enzyme at the beginning of the stationary phase has also been reported earlier[17]. A maximum enzyme activity of 1.65 IU mg^{-1} dry weight was observed after 24 h of incubation. The results are in agreement with earlier researchers such as Ku and Hang[13] who have reported 24 h as an optimal incubation period. Similarly, Ranzi *et al.*[18] have reported the optimum incubation time of 21 h using *K. lactis,* whereas, a fermentation time of 18.5 h has also been reported optimal using *K. lactis*[11].

Keeping these observations in view, the incubation time of 24 h was considered optimal for maximum β-D-galactosidase activity under the above optimized conditions and used in further studies.

From the foregoing account and the observations made during the process optimization studies, it can be concluded that maximum enzyme activity can be obtained with the process conditions of pH 5.0, temperature 30°C and inoculum size 6% (v/v) of 20 h old yeast culture under shaking conditions (100 rpm) for an incubation of 24 h. The different optimal conditions reported by various researchers for maximum β-D-galactosidase activity could be explained by the differences in the nature of the strains and medium composition used in their studies.

ACKNOWLEDGMENT

The authors are thankful to MHRD, New Delhi, Government of India for their financial support to carry out this study.

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