Production of β -galactosidase by *Kluyveromyces marxianus* MTCC 1388 using whey and effect of four different methods of enzyme extraction on β -galactosidase activity

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Abstract Whey containing 4.4% (w/v) lactose was inoculated with *Kluyveromyces marxianus* MTCC 1389 for carrying out studies related to β -galactosidase production. β -galactosidase activity was found to be maximum after 30 h and further incubation resulted in decline in activity. The maximum cell biomass of 2.54 mg mL⁻¹ was observed after 36 h of incubation. Lactose concentration dropped drastically to 0.04 % from 4.40% after 36 h of incubation. Out of the four methods tested for extraction of enzyme, SDS – Chloroform method was found to be best followed by Toluene – Acetone, sonication and homogenization with glass beads in that order. It could be concluded through this study that SDS – Chloroform is cheap and simple method for enzyme extraction from *Kluyveromyces* cells, which resulted in higher enzyme activity as compared to the activity observed using the remaining extraction methods. The study could also establish that whey could effectively be utilized for β -galactosidase production thus alleviating water pollution problems caused due to its disposal into the water streams.

Keywords Whey · β -galactosidase activity · Lactose · Biomass · SDS- Chloroform

Introduction

β -galactosidase or lactase is one of the important enzymes used in dairy industry for hydrolysis of lactose into glucose and galactose. Although there is a large variation in the lactose intolerant population in different countries of the world, on an average about 70% population of the world suffers from this problem, which makes the importance of enzyme even more pronounced. The need for low-lactose milk is particularly important in food-aid programmes as severe tissue dehydration, diarrhoea and even death may result from feeding lactose containing milk to lactose - intolerant children and adults suffering from protein-calorie malnutrition. Besides, lactose has a low solubility resulting in crystal formation at concentrations above 11 % (w/v), which prevents the use of concentrated whey syrups in many food processes.

India is a leading producer of milk in the world and about 2 and 1.5 million tones of channa and paneer (cottage cheese) respectively are produced annually [1] and during their production about 75–85% of the volume of milk is removed as whey. Whey has immense therapeutic applications due to its composition in terms of proteins, lactose and minerals and contains about 6.66% of valuable milk nutrients [2]. The disposal of whey remains a significant problem for the dairy industry especially in developing countries where a relatively insignificant part of whey is used for production of whey protein concentrates or permeates and a significant part of it is disposed off into the water streams causing serious water pollution problems resulting from high BOD [3] mainly due to the presence of 5–6% dissolved solids of which lactose is the main constituent. The main options available for treatment or bioconversion of whey into commercially important products could be the

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use of whey permeate as an inexpensive feedstock for ethanol production [3] or production of β -galactosidase which finds an increasing use because of growing lactose intolerant population. However, compared with the production of ethanol from glucose by traditional *Saccharomyces cerevisiae* strains, organisms fermenting lactose exhibit inferior production rates and yields [4]. Since, β -galactosidase is an intracellular enzyme, one of the major hindrances in effective production of β -galactosidase is the release of enzymes in sufficient quantities from the cells. Although the use of whole cells as a source of β -galactosidase may appear as a good alternative, a major drawback in the use of whole cells is the poor permeability of cell membrane. Different methods such as sonication, use of CTAB, Osgall, Triton X 100, mixture of SDS – Chloroform, physical disruption etc have been reported in literature [5], it is important to evaluate the ideal method in terms of efficacy, cost and enzyme yield so that the process could be scaled up to pilot scale and further commercial levels. Although different microbes such as *Streptococcus thermophilus*, *E. coli*, *Candida pseudotropicalis* and *Kluyveromyces* sp have been studied for production of β -galactosidase at shake flask level [6–8], yeasts being safe have been extensively exploited for β -galactosidase production. Thus, the present study was conducted to evaluate the best method for β -galactosidase extraction from *Kluyveromyces marxianus* cells as strains of *Kluyveromyces* are known for their enzyme production capabilities from whey [9, 10, 11].

Materials and methods

Whey was procured from a local dairy and analysed for lactose content. *Kluyveromyces marxianus* MTCC 1388, was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India and was grown on sterilized malt yeast extract medium containing (g L⁻¹): malt extract, 3.0; yeast extract, 3.0; peptone, 5.0 and glucose, 10. The cultures were incubated at 28°C for 48 h and maintained at 4°C on agar slants and sub cultured fortnightly. A loopful of culture from the slants mentioned above was inoculated into sterilized 50 mL of Malt yeast extract broth in 150 mL flasks and incubated in an incubator shaker at 100 rpm and 30°C for about 24 h or more depending upon the cell count observed in each case which served as a starter culture.

Extraction methods used for the study

Sonication: β -galactosidase extraction was done by using the method of Beccerra *et al* [12] using Soniprep 150 MSE for release of enzyme. The cells were sonicated using 9 mm

probe in the sonicator at 16 microns for 20 min with intervals of 10, 5 and 5 min at 4°C and the sonicate containing cell debris was centrifuged at 20,000 rpm at 4°C for one hour. The supernatant cell free extract was stored at –20°C for enzyme assays.

Toluene – Acetone method: 10 mL sample was centrifuged at 8000 rpm at 5°C (Eltek Mp–400 R) and the pellet obtained was dissolved in 2 mL, 0.2 M phosphate buffer (pH 7.0), ground with 2.0 g alumina and 0.1 mL toluene : acetone (9:1) solution in a pestle and mortar. (Mahoney *et al* [13] with slight modification.). The suspension was redissolved in 8 mL buffer and centrifuged at 12,000 rpm at 5°C for 10 min. The supernatant obtained was used for enzyme assay.

SDS - Chloroform method: 2 mL sample was centrifuged at 8000 rpm at 5°C for 10 min and the pellet obtained was re-suspended in 2 mL of the chilled Z buffer (composition per 50 ml: 0.8g Na₂HPO₄·7H₂O, 0.28g NaH₂PO₄·H₂O, 0.5 mL 1M KCl, 0.05mL 1M MgSO₄, 0.135mL β -mercaptoethanol, pH-7.0). 0.1 mL of the suspension was again mixed with 0.9 mL Z buffer and permeabilization was done by addition of 100 μ L chloroform and 50 μ L 0.1 % SDS solution (Miller [14] with slight modification). The suspension was centrifuged at 5000 rpm for obtaining a clear supernatant (cell free extract) which was used for enzyme assay.

Homogenization: 10 mL of the sample was centrifuged at 8000 rpm at 5°C and the pellet obtained was dissolved in 10 mL 0.2 M phosphate buffer (pH 7.0), cells were broken by shaking with an equal volume of glass beads (0.45–0.50 mm diameter) for 1 min in a warring blender. The homogenate was extracted with an equal volume of the same phosphate buffer for 4 h at 4°C and then centrifuged to remove cell debris [13]. The supernatant obtained was used for determination of enzyme activity.

β -galactosidase production: 200 mL sterilized whey in each of the 500 mL Erlenmeyer flasks was inoculated @ 10% (v/v) with the prepared starter culture by standardizing the initial cell count at 1×10^7 cells mL⁻¹. The flasks were incubated at 28°C in an incubator shaker at 100 rpm. Initial pH in each case was set at 5.0 using 1N HCl or 1N NaOH prior to sterilization. The samples were drawn at 6 h interval and analysed for β -galactosidase activity, biomass and protein content. The extraction method which resulted in highest enzyme production was used for the rest of the experiments. The experiment was planned in simple CRD in triplicate and the data was analysed using ANOVA.

Analytical methods: Lactose content in whey was determined by the method prescribed by Nickerson *et al* [15] and the protein content in the supernatant was analysed by the method of Bradford [16]. One unit of enzyme activity

was defined as the quantity of the enzyme that catalysed the release of one micromole of orthonitrophenol from ONPG per minute under standard assay conditions. All the colorimetric estimations were done using microprocessor based double beam UV-VIS spectrophotometer (Spectroscan DV 80). The specific activity was calculated in terms of enzyme unit produced per mg of protein. The cell biomass was separately determined by centrifuging the known volume of samples at 5,000 rpm at 4°C and the pellet was used for biomass estimation. The contents of the pellet were washed and dried on the pre weighed Whatman No.1 filter paper using precision balance (Citizen CX 120) and the difference in the weight was calculated as weight of the cells or biomass and calculated as mg mL⁻¹.

Results and discussion

Impact of extraction methods on β -galactosidase activity:

It is clear from Table 1 that Chloroform – SDS method of cell permeabilization was found to be best amongst the four methods tried for enzyme extraction. The enzyme activity using the SDS-Chloroform method of extraction was found to be nearly 47, 71 and 223% higher than the remaining methods i.e. Toluene-Acetone + Alumina method, sonication and homogenization methods respectively. This is well reflected in the case of specific activity too primarily due to β -galactosidase activity, although the initial total cell biomass was same in all the cases. Although sonication is a preferred method in case of bacteria, Beccera *et al* [12] had indicated that sonication was found to be most effective method of cell enzyme extraction in case of *Kluyveromyces lactis* which is in contrast with our results. Mahoney *et al* [13] used concentrated whey containing 15% lactose and observed the β -galactosidase activity in the range of 0.039–2.45 U mg⁻¹ using the homogenization with glass beads method. Available literature suggests that chemical methods are ideal for enzyme extraction especially from yeast cells [5]. Although the Toluene – Acetone – Alumina method was effective in release of enzymes, it was not as

effective as the SDS- Chloroform method. SDS is a non ionic detergent which works by disrupting non-covalent bonds in the proteins, thereby denaturing them, causing the molecules to lose their native shape (conformation). Chloroform on the other hand is a common solvent because it is relatively unreactive, miscible with most organic liquids, and conveniently volatile. Chloroform is an effective solvent for alkaloids in their base form and thus plant material is commonly extracted with chloroform for pharmaceutical processing. Thus the action of SDS and chloroform could be of synergistic nature resulting in efficient permeabilization of cell wall of yeast cells and subsequent release of the enzyme. Homogenization with glass beads is a mechanical and crude method of enzyme extraction and thus it is quite possible that effective disruption of all the cells could not be done. Thus, it is clear that the SDS-Chloroform method besides being simple could be quite economical too if the process of β -galactosidase from whey is scaled up at a commercial level due to reasonable enzyme activity. Our results are comparable or even better as compared to results obtained by Panesar *et al.* [8] and Sonawat *et al.* [17] in terms of complexity of the process, ease of operations and economics since only whey was used as a substrate for enzyme production. Thus the SDS – Chloroform method was used for enzyme extraction from *Kluyveromyces* cells for future experiments.

β -galactosidase production at different time intervals: As indicated by Fig. 1, irrespective of the method of extraction, the maximum β -galactosidase activity was observed after 30 h of incubation, beyond which a decline in activity was observed. The trend observed is mainly due to the physiological changes during growth of the cells and subsequent changes in the media and environment during metabolism of the cells. Our results are in partial agreement with the results reported earlier [8] where maximum enzyme activity was observed at 24 h of incubation using 5% lactose and different nitrogenous sources but the authors reported a slow decline on this account with incubation time. However, our results are in line with the results

Table 1 Effect of different methods of extraction on β -galactosidase activity after 30 h of incubation

Methods	β -galactosidase activity (U mg ⁻¹)	Specific activity (U mg protein ⁻¹)
SDS -Chloroform	1.68	59
Sonication	0.98	35
Toluene – Acetone + Alumina	1.14	41
Homogenization using glass beads	0.52	21
CD (0.05)	0.05

Fig. 1 Effect of incubation time on lactose utilization and cell biomass

Fig. 2 Effect of different methods of enzyme extraction on β -galactosidase activity at different time intervals

reported by Mahoney *et al.* [13]. Maximum β -galactosidase activity at about 22 h for most of the trials on growth of the organism and enzyme production under different air pressure has also been reported [18]. Some authors have also reported maximum enzyme activity even after 12 h using different media [17] but most of the available literature suggests the optimal fermentation time in the range of 20–36 h [13, 19, 20]. Maximum enzyme activity at the beginning of the stationary phase has also been reported earlier [21].

Effect of incubation period on cell biomass and lactose utilization: Lactose content in whey determined by the method mentioned above was found to be 4.4% (w/v). Fig. 2 indicates an increase in biomass till about 36 h and further

increase in incubation period resulted in no further increase in the biomass, rather a marginal decline could be observed during the extended growth phase. This could probably be due to nutrient depletion and physiological changes occurring in the medium and environment during extended growth of the cells resulting in cells going into the stationary phase and eventually death of few cells. Further, the biomass could be maintained at a stationary level for some time due to presence of live cells and sugars in the medium. Our results are corroborated by the observations made earlier [13]. The rate of lactose consumption increased drastically with time initially but the same decreased after 24 h and subsequently touched the axis. This is primarily due to the lactose utilization by cells at different growth stages. Lactose might have been initially consumed for biomass build up and since it acts as an inducer for β -galactosidase; enzyme production could have started immediately at the end of lag phase. Since the enzyme breaks down lactose into fermentable glucose and galactose resulting in production of ethanol subsequently, most of the lactose in whey was consumed just after 24 h and its concentration became almost nil at 36 h of incubation. Similar trend has been observed during β -galactosidase production using concentrated whey and different *K. fragilis* strains [13]. Most of the available literature on ethanol production from whey suggests a drastic reduction in lactose content during growth of cells and subsequent fermentation [22, 23].

Conclusion

This study could establish that whey which contributes significantly to the environmental pollution due to its poor disposal into the water streams could alone be efficiently used for β -galactosidase production using *Kluyveromyces marxianus*. Thus whey could easily be discharged into water streams after being exploited for commercial production of β -galactosidase. The SDS-Chloroform method of enzyme extraction has been found to be simple, cheap and effective for cell permeabilization and release of enzymes from *Kluyveromyces marxianus*. The major factor contributing to the higher BOD in water streams due to its discharge into such streams is lactose whose concentration became nearly nil at the time of maximum enzyme activity.

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