

Utilization of Whey for the Production of β -Galactosidase Using Yeast and Fungal Culture

Rupinder Kaur, Parmjit S. Panesar, Ram S. Singh

Abstract—Whey is the lactose rich by-product of the dairy industry, having good amount of nutrient reservoir. Most abundant nutrients are lactose, soluble proteins, lipids and mineral salts. Disposing of whey by most of milk plants which do not have proper pre-treatment system is the major issue. As a result of which, there can be significant loss of potential food and energy source. Thus, whey has been explored as the substrate for the synthesis of different value added products such as enzymes. β -galactosidase is one of the important enzymes and has become the major focus of research due to its ability to catalyze both hydrolytic as well as transgalactosylation reaction simultaneously. The enzyme is widely used in dairy industry as it catalyzes the transformation of lactose to glucose and galactose, making it suitable for the lactose intolerant people. The enzyme is intracellular in both bacteria and yeast, whereas for molds, it has an extracellular location. The present work was carried to utilize the whey for the production of β -galactosidase enzyme using both yeast and fungal cultures. The yeast isolate *Kluyveromyces marxianus* WIG2 and various fungal strains have been used in the present study. Different disruption techniques have also been investigated for the extraction of the enzyme produced intracellularly from yeast cells. Among the different methods tested for the disruption of yeast cells, SDS-chloroform showed the maximum β -galactosidase activity. In case of the tested fungal cultures, *Aureobasidium pullulans* NCIM 1050 was observed to be the maximum extracellular enzyme producer.

Keywords— β -galactosidase, fungus, yeast, whey.

I. INTRODUCTION

THE enzyme lactase, trival name of β -galactosidase (E.C. 3.2.1.23), has been classified under the glycoside hydrolase family of the enzymes depending upon their substrate specificity [1]. The ability of the enzyme to catalyze the hydrolysis and transgalactosylation reactions for the production of monosaccharides (glucose, galactose) as well as prebiotics (galactooligosaccharides, lactulose) have potential applications in food, pharmaceutical as well as biotechnology industries [2] [3]. The hydrolysis of lactose has many benefits as it provides alternatives for the lactose intolerant people who show lactose intolerance [4]. Moreover, the lactose hydrolyzed milk could be used in the production of frozen dairy products such as ice-creams, condensed milks and it could also help to

solve the environmental problems related with the disposal of whey [5].

β -galactosidase can be extracted from various sources such as plants, animals and microorganisms [6]. Microorganisms are much investigated for the production of β -galactosidase as higher yields are obtained when compared with plants and animals. Moreover, seasonal variation has no effect on the production [7]. The most common microorganisms that are utilized for the production of the enzyme are *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus circulans*, *Escherichia coli* and *Lactobacillus bulgaricus* [8], however the properties, stability and specificity of the enzyme varies with source of the enzyme [9].

Among different microorganisms, yeast has been reported to produce intracellular enzyme with respect to the fungus that are extracellular in nature [10]. Thus, yeast cells have to be disrupted for the extraction of intracellular enzyme by different disruption techniques such as physical and chemical methods [11].

Whey is the aqueous by-product of the cheese production remaining after the coagulation of milk and removal of casein [12]. The main components of whey include lactose, protein, lipids and mineral matter, with lactose (4.5-5%) being the major component [13]. The high percent of lactose present in whey increases its biological and chemical oxygen demand, which creates problem in the disposal of whey [14]. The high lactose content in the whey acts as a carbon source for the growth of the microorganisms as well as an inducer for production of the enzyme [15]. The use of cheap substrate and efficient strains can enhance the economy of the process for the production of the enzyme.

The present study was carried for the production of β -galactosidase utilizing whey as a substrate from both fungal and yeast cells. The extraction of intracellular enzyme from the yeast cells was done by using different disruption techniques.

II. MATERIALS AND METHODS

A. Isolation and Procurement of Microbial Cultures

1. Isolation of Yeast Strain

Yeast *Kluyveromyces marxianus* WIG2, was isolated from the whey procured from the milk industry, India.

2. Procurement of Fungal Cultures

Three fungal cultures, viz., *Aureobasidium pullulans* NCIM 1050, *Aspergillus oryzae* NCIM 1212, *A. niger* NCIM 616

S. B. Parmjit S. Panesar and F.A. Rupinder Kaur are with the Food Biotechnology Research Laboratory, Department of Food Engineering & Technology, Sant Longowal Institute of Engineering & Technology, Longowal 148 106, Punjab, India (corresponding author the provide phone: +91-1672-253252; Fax: +91-1672-253531, e-mail: pspbt@yahoo.com, 29.rupinderkaur@gmail.com).

T. C. Ram S. Singh is with Biotechnology Department, Punjabi University, Patiala 147002, Punjab, India (e-mail: rssingh11@lycos.com)

have been procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. *Aspergillus flavus* MTCC 9349 has been procured from Microbial Type Culture Collection Centre, Institute of Microbial Technology, Chandigarh, India for the production of β -galactosidase.

B. Physico-Chemical Analysis of Whey:

The physico-chemical characterization of whey for different parameters such as pH, lactose concentration, total protein, fat and solid content were examined by standard methods.

C. Inoculum Preparation of Microbial Cultures

Loopful of yeast maintained on agar slants was added to the media containing 1% glucose, 0.3% malt extract, 0.3% yeast extract and 0.5% peptone at pH 5. The incubation was done at 30°C for 20 h.

The fully grown fungal spores on potato dextrose agar (PDA) slants were suspended in 10 mL of distilled water.

D. Production of β -Galactosidase from Yeast Cells

The fermentation medium for the production of β -galactosidase from yeast cells constituted of whey supplemented with 1.7% corn steep liquor, 0.39% yeast extract and 0.05% magnesium sulphate at pH 5.2. The fermentation was carried for 20 h at 30°C.

E. Production of β -Galactosidase from Fungal Cells

The fermentation media for the production of β -galactosidase comprised of whey supplemented with 0.5% yeast extract. The fermentation media was inoculated with 2% spore suspension and incubation was carried for 7-8 days at 28°C. The samples were withdrawn regularly after 24 h for the determination of enzyme activity.

F. Determination of Enzyme Activity

Yeast β -galactosidase activity assay was carried following the method of Miller [16].

The enzyme assay for fungus was carried out by following the method of [17]. The culture was centrifuged at 5000 rpm for 10 min. The supernatant was used for enzymatic assay. 0.2 mL of the enzyme solution was mixed with the 1 mL ONPG in 0.1M sodium acetate buffer (pH 4.5). The enzyme solution along with the substrate was incubated at 50°C for 5 min. The reaction was stopped by using 1 mL of 10% sodium carbonate and the absorbance was read at 420 nm (DR 5000, HACH, Germany).

One unit of enzyme activity is equivalent to 1 micromole of ortho-nitrophenol liberated per min. under standard assay conditions.

G. Cell Disruption Techniques

Different physical methods (homogenization in the bead mill, grinding with river sand, sonication, freeze-thaw) and chemical methods (SDS-Chloroform, toluene, iso-amyl alcohol, acetone) were investigated for the extraction of β -galactosidase from yeast cells. The techniques were carried by following the methods of [18]; [19], along with some modifications.

1. Homogenization in a Bead Mill

Fermentation broth was centrifuged (Remi C-24BL) at 4000 rpm for 10 min, and the pellet obtained was suspended in the phosphate buffer (pH 7). The suspension was homogenized in a bead mill using silica beads (0.5 mm dia.) for 80-90s. The disrupted cells were separated by centrifugation and the supernatant was taken for the enzyme assay.

2. Grinding with River Sand

Pretreatment of the river sand was done by boiling it in the distilled water followed by washing twice in diethyl ether, once in absolute alcohol and further drying in the oven. The pellet obtained after centrifugation was suspended in phosphate buffer (pH 7). The suspension was mixed with river sand (1g/10 mL of cell suspension). The pre-chilled mixture was grounded in the pestle and mortar for 15 min, after which 50 mL of phosphate buffer was added. The sand and the disrupted cell were removed after centrifugation; the supernatant was taken for enzyme activity.

3. Sonication

The pellet obtained after centrifugation was suspended in 10 mL of phosphate buffer (pH 7). The suspended cells were disrupted in the sonicator (Cole Parmer CPX 500) for 160s with alternate periods of cooling. The enzyme activity of the supernatant was carried after centrifugation of the disrupted cells.

4. Freeze-Thaw

The culture broth was centrifuged at 4000 rpm for 10 min. The pellet obtained after centrifugation was suspended in the equal quantities of phosphate buffer (pH 7). The cells were subjected to alternate freezing and thawing cycles at -18°C and 4°C, respectively. The cell debris was removed by centrifugation and the supernatant was taken for enzyme assay.

5. SDS-Chloroform

An aliquot of suspended cells (1 mL) diluted with Z-buffer, (composition (gL⁻¹): Na₂HPO₄.7H₂O, 16.1; NaH₂PO₄.H₂O, 5.5; KCl, 0.75; MgSO₄.H₂O, 0.246 and β -mercaptoethanol 2.7mL) was mixed with different concentrations of SDS-Chloroform to find out the optimum concentration of the mixture required for cell disruption. Further, incubation of the assay was carried at different time intervals at 30°C to find out the optimum treatment time for maximum β -galactosidase activity.

6. Toluene

The culture broth was centrifuged and the pellet obtained was suspended in the same quantity of the phosphate buffer (pH 7). An aliquot of suspended cells mixed with 1 mL of pre-chilled toluene was incubated at 30°C for 15 min. The mixture was centrifuged and the supernatant was assayed for enzyme activity.

7. Iso-Amyl Alcohol

The suspended pellet was mixed with 0.85 mL iso-amyl alcohol and the volume was made to 5 mL with phosphate buffer. After the incubation for 15 min., the cells were centrifuged and the supernatant was taken for enzymatic assay.

8. Acetone

1 mL of the suspended cells was mixed with 5 mL of acetone. After incubation for 15 min at 30°C, centrifugation was carried at 5000 rpm for 15 min. The disrupted cells were removed as a pellet and the supernatant was taken for the determination of enzyme activity.

III. RESULTS AND DISCUSSION

The efficiency of yeast and fungal strains for the production of β -galactosidase has been tested and the results have been discussed in the following sections.

A. Physico-Chemical Characterization of Whey

The physico-chemical analysis of whey showed that it contained high amount of lactose ($4.89 \pm 0.11\%$, w/v), followed by protein ($0.48 \pm 0.33\%$, w/v), fat ($0.18 \pm 0.08\%$, w/v) and traces of minerals as shown in Table I.

Parameters	Results
Lactose	$4.89 \pm 0.11\%$ (w/v)
Total protein content	$0.48 \pm 0.33\%$ (w/v)
Total fat content	$0.18 \pm 0.08\%$ (w/v)
Total solid content	$7.1 \pm 0.13\%$ (w/v)
pH	6.15 ± 0.06

B. Production of β -Galactosidase from Yeast Cells

The production of the enzyme β -galactosidase was carried by using novel yeast isolate *Kluyveromyces marxianus* WIG2 utilizing whey as a substrate under the optimized conditions. Due to the intracellular location of the enzyme, the different cell disruption techniques have been studied for the extraction.

C. Cell Disruption Techniques

Different physical (homogenization in the bead mill, grinding with river sand, sonication, freeze-thaw) and chemical methods (SDS-chloroform, toluene, iso-amyl alcohol, acetone) were used for the extraction of intracellular β -galactosidase. Among the physical disruption techniques, maximum cells were disrupted by homogenization in the bead mill with an enzyme activity of 1650 IU/g DW, followed by sonication as shown in Fig. 1. Enzyme activity of 376 IU/gDW and 508 IU/g DW were obtained by disruption using river sand and freeze-thaw, respectively.

Among the different chemical methods tested for the disruption of the yeast cells, SDS-chloroform showed the maximum enzyme activity (2886 IU/gDW) followed by toluene (2723 IU/gDW) as shown in Fig. 2. The disruption of the cells carried by iso-amyl alcohol and acetone showed an

enzyme activity of 2510 IU/gDW and 1840 IU/gDW, respectively.

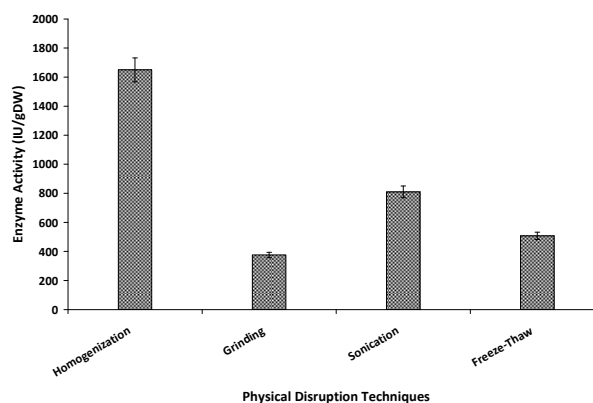


Fig. 1 Effect of physical disruption techniques on the enzyme activity

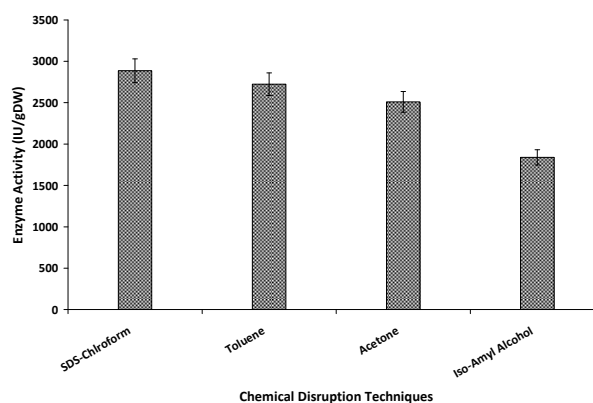


Fig. 2 Effect of chemical disruption techniques on the enzyme activity

The concentrations of SDS-Chloroform were varied to study the optimum concentration required for the disruption of the cells. Further, the optimum treatment time required by the enzyme β -galactosidase to complete the reaction was also studied. The maximum enzyme activity (2765 IU/gDW) was observed using SDS:Chloroform in the mixture of 200:500 μ L/mL of enzyme (Fig. 3), whereas the treatment time of 10 min. showed a maximum enzyme activity of 2669 IU/gDW as depicted in Fig. 4.

Different methods of cell disruption have also been investigated by [20] for the isolation of β -galactosidase from *Kluyveromyces marxianus* MTCC 1388. Among all the techniques, they indicated SDS-chloroform as the suitable method of extraction as compared to the other techniques. SDS is a non-ionic detergent that breaks the non-covalent bond of the proteins; thereby changing their native structure. Chloroform is a common solvent which is non-reactive and miscible with the other solvents. Thus the action of both SDS and chloroform helps in the lysis of the cell wall and extraction of the enzyme [21].

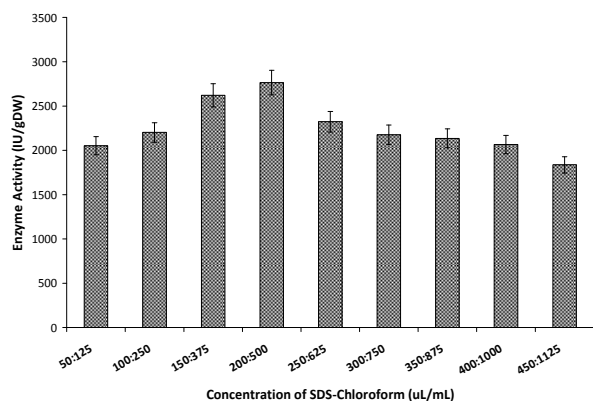


Fig. 3 Effect of SDS-Chloroform concentration on the enzyme activity

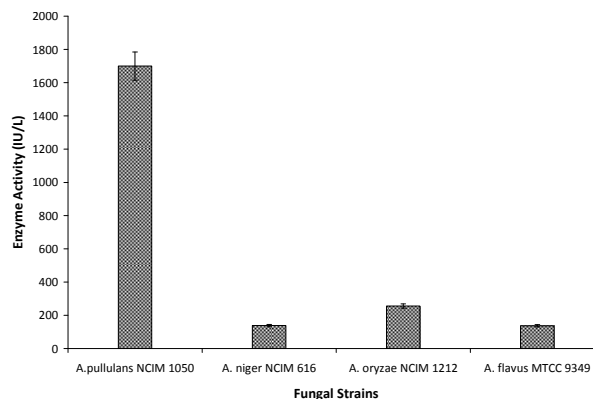


Fig. 5 Screening of the different fungal strains for β -galactosidase production

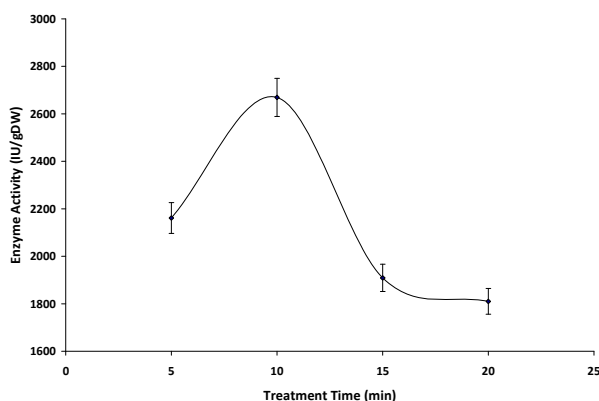


Fig. 4 Effect of treatment time on the enzyme activity

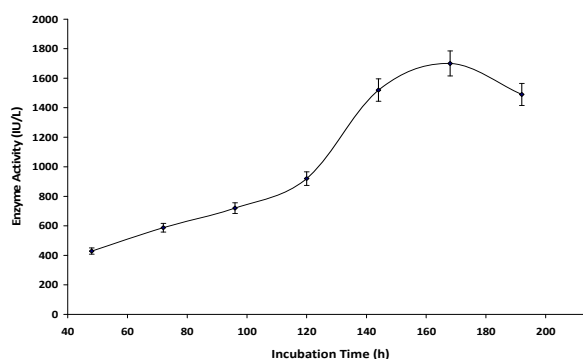


Fig. 6 β -galactosidase production by *Aureobasidium pullulans* NCIM 1050 as a function of incubation time

D. Screening of the Fungal Strains for β -Galactosidase Production

Different fungal cultures such as *Aspergillus flavus* MTCC 9349, *A. oryzae* NCIM 1212, *A. niger* NCIM 616 and *Aureobasidium pullulans* NCIM 1050 were investigated for the production of β -galactosidase utilizing whey as a substrate. The submerged fermentation was carried for 7-8 days and the sample was withdrawn regularly at 24 h interval for the enzymatic assay of β -galactosidase. *Aureobasidium pullulans* NCIM 1050 showed the maximum enzyme activity of 1700 IU/L followed by *A. oryzae* NCIM 1212 as shown in Fig. 5. *A. flavus* MTCC 9349 and *A. niger* NCIM 616 produced the low levels of β -galactosidase. Similar findings were observed during the production of β -galactosidase from different fungal strains utilizing whey [22].

E. Effect of Incubation Time on Enzyme Production

The production of β -galactosidase from *Aureobasidium pullulans* was studied with respect to incubation time.

The samples were withdrawn at 24 h interval for the determination of the maximum enzyme production. The enzyme production was found to increase with the increase in incubation time. The maximum production was observed during the 168 h (7th day) of fermentation as shown in Fig. 6.

IV. CONCLUSIONS

Yeast and fungal strains have been tested for their β -galactosidase production potential utilizing whey as a substrate. Different methods have been employed for the disruption of the *Kluyveromyces marxianus* cells for the extraction of intracellular β -galactosidase enzyme. Among the different physical and chemical methods, SDS-Chloroform was observed to be the best method for yeast cell disruption with a maximum enzyme activity of 2886 IU/gDW. In case of fungal strains, *Aureobasidium pullulans* NCIM 1050 was found to be an efficient β -galactosidase producer with an enzyme activity of 1700 IU/L after incubation period of 168 h.

ACKNOWLEDGMENT

The authors acknowledge the financial support given by Department of Biotechnology (DBT), New Delhi, India to carry out this work.

REFERENCES

- [1] A. C. Adam, M. Rubio-Teixeira, J. Polaina, "Lactose: the milk sugar from a biotechnological perspective," *Crit. Rev. Food Sci. Nutr.*, vol. 44, pp. 553- 557, Dec. 2010
- [2] P. S Panesar, S. Kumari, R. Panesar, "Potential applications of immobilized β -galactosidase in food processing industries". *Enzyme Res.*, vol, 2010, pp. 1-16, Dec. 2010.

- [3] S. Kumari, P. S. Panesar, M. B. Bera, B. Singh, "Permeabilization of yeast cells for β -galactosidase activity using a mixture of organic solvents: A response surface methodology approach," *Asian J. Biotechnol.*, vol. 3, pp. 406-414, June 2011.
- [4] A. O. Johnson, J. G. Semenya, M. S. Buchowski, C. O. Enwonwu, N. S. Scrimshaw, "Correlation of lactose maldigestion, lactose intolerance and milk intolerance," *Am. J. Clin. Nutr.*, vol. 57, pp. 199-401, March 1993.
- [5] M. Becerra, B. Barolib, A. M. Fadda, J. B. Méndez, M. I. González-Siso, "Lactose bioconversion by calcium-alginate immobilization of *Kluyveromyceslactis* cells," *Enzyme Microb. Technol.*, vol. 29, pp. 506-512, Nov. 2001.
- [6] T. Haider, Q. Husain, "Calcium alginate entrapped preparations of *Aspergillusoryzae* beta galactosidase: its stability and applications in the hydrolysis of lactose," *Int. J. Biol. Macromol.*, vol. 41, pp. 72-80, June 2007b.
- [7] A. Santos, M. Ladero, F. Garcia-Ochoa, "Kinetic modelling of lactose hydrolysis by a β -galactosidase from *Kluyveromyces fragilis*," *Enzyme Microb. Technol.*, vol. 22, pp. 558-567, May 1998.
- [8] S. A. Shaikh, J. M. Khire, M. I. Khan, "Production of β -galactosidase from thermophilic fungus *Rhizomucor* sp," *J. Ind. Microbiol. Biotechnol.*, vol. 19, pp. 239-245, Oct. 1997.
- [9] Q. Z. K. Zhou, X. D. Chen, "Effects of temperature and pH on the catalytic activity of the immobilized β -galactosidase from *Kluyveromyceslactis*," *Biochem. Eng. J.*, vol. 9, pp. 33-40, Nov. 2001.
- [10] R. E. Huber, M. N. Gupta, S. K. Khare, "The active site and mechanism of the β -galactosidase from *Escherichia coli*," *Int. J. Biochem.*, vol. 26, pp. 309-318, March 1994.
- [11] F. Zhao, J. Yu, "L-Asparaginase release from *Escherichia coli* cells with K_2HPO_4 and triton X-100," *Biotechnol. Progress*, vol. 17, pp. 490-494, June 2001.
- [12] C. Spălăţelu, "Biotechnological valorisation of whey," *Innov. Rom. Food Biotechnol.*, vol. 10, pp. 1-8, March 2012.
- [13] F. V. Kosikowski, "Whey utilization and whey products," *J. Dairy Sci.*, vol. 62, pp. 1149-1160, July 1979.
- [14] S. S. Marwaha, J. F. Kennedy, "Review: whey-pollution problem and potential utilization," *Int. J. Food Sci. Technol.*, vol. 23, pp. 323-336, Aug. 1988.
- [15] H. M. Sonawat, A. Agrawal, S. M. Dutta, "Production of β -galactosidase from *Kluyveromyces fragilis* grown on whey," *Folia Microbiol.*, vol. 26, pp. 370-376, Feb. 1981.
- [16] J. H. Miller, "Experiments in molecular genetics," Cold Spring Harbor Laboratory, New York, 1972, pp. 352.
- [17] K. Reczey, H. Stalbrand, B. Hahn-Hegerdal, F. Tijernal, "Mycelia-associated β -galactosidase activity in microbial pellets of *Aspergillus* and *Penicillium* strains," *Appl. Microbiol. Biotechnol.*, vol. 38, pp. 393-397, Dec. 1992.
- [18] M. Puri, S. Gupta, P. Pahuja, A. Kaur, J. R. Kanwar, J. F. Kennedy, "Cell disruption optimization and covalent immobilization of β -galactosidase from *Kluyveromyces marxianus* YW-1 for lactose hydrolysis in milk," *Appl. Biochem. Biotechnol.*, vol. 160, pp. 98-108, Jan. 2010.
- [19] A. M. Gupte, J. S. Nair, " β -galactosidase production and ethanol fermentation from whey using *Kluyveromyces marxianus* NCIM 3551," *J. Sci. Ind. Res.*, vol. 69, pp. 855-859, Nov. 2010.
- [20] S. Bansal, H. S. Oberoi, G. S. Dhillon, R. T. Patil, "Production of β -galactosidase by *Kluyveromyces marxianus* MTCC 1388 using whey and effect of four different methods of enzyme extraction on β -galactosidase activity," *Indian J. Microbiol.*, vol. 48, pp. 337-341, Sep. 2008.
- [21] P. S. Panesar, R. Panesar, R. S. Singh, J. F. Kennedy, H. Kumar, "Microbial production, immobilization and applications of β -galactosidase," *J. Chem. Technol. Biotechnol.*, vol. 81, pp. 530-543, Apr. 2006.
- [22] R. R. Saad, "Purification and some properties of β -galactosidase from *Aspergillus japonicas*," *Ann. Microbiol.*, vol. 54, pp. 299-306, 2004.