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Qualitative analysis of galacto-oligosaccharides produced by free and immobilized enzyme by thin layer chromatography

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Abstract
Galactooligosaccharides (GOS) are the non-digestible oligomers of galactose linked to β terminal end of glucose or galactose and are recognized as functional food ingredients. GOS are produced by transgalactosylation reaction mediated by β-galactosidase enzyme where galactosyl residues are transferred to lactose molecules. Generally, β-galactosidase suffers from loss of catalytic activity at high substrate concentration, instability to severe environmental conditions and no subsequent reuse. It can be overcome by immobilizing the enzyme on proper support materials. In the present study, the β-galactosidase was immobilized on zinc oxide nanoparticles (ZnO NPs) and evaluated its effect on production of GOS at different enzyme concentration. The GOS content was determined using thin layer chromatography analysis. The study revealed that GOS content was observed above 0.1% concentration of both free and nano-immobilized enzymes. With increase in the concentration of both enzymes, the concentration of GOS was increased. However, the samples treated with immobilized enzyme (at lower concentration) showed thicker GOS bands indicates higher efficiency of GOS production.

Keywords: Galactooligosaccharides (GOS), immobilized enzyme, layer chromatography

Introduction
β-Galactosidase is one of the most widely used industrial enzymes due to its capability to hydrolyse lactose. This enzyme can be obtained from various microbial sources such as bacteria, fungi or yeast with variable properties depending on the species. It has been recommended as a key ingredient to hydrolyze lactose in several milk-based food products for consumption by lactose intolerant individuals. By transferring galactosyl residues to lactose molecules in the presence of highly concentrated lactose, this enzyme can produce galactooligosaccharides (GOSs) (Gibson *et al.*, 2017) [7]. β-galactosidase enzyme can be immobilized on the proper support material to enhance the catalytic activity, stability to severe environmental conditions and subsequent reuse. To economically enable the use of immobilized enzymes, one must consider the type of support used and the method of immobilization, which highly influence the activity and subsequent reuse of the biocatalyst (Pessela *et al*., 2007; Villeneuve *et al*., 2000) [10, 14]. Hence, immobilization of enzymes on nanoparticles appears to be more prominent and could be useful for building continuous production systems. Hence, in the present investigation, the activated zinc oxide (ZnO) nanoparticles are proposed to be used for immobilization of β-galactosidase and qualitative analysis of galacto-oligosaccharides is carried out by Thin layer chromatography (TLC).
The TLC technique is commonly used to separate mixtures having different molecular weight. Compound separation is based on the competition between the solute and mobile phase for binding sites on the stationary phase (Bele and Khale, 2011) [2]. Due to the polarity variations of the analytes, separation is accomplished on a "stationary phase" (often silica gel) (Fuchs *et al*., 2011) [6]. A higher Rf value is produced by the less polar molecule moving up the plate. All compounds on the TLC plate will rise higher up the plate if the mobile phase is changed to a more polar solvent or solvent mixture because it is better able to dislodge solutes from the silica binding sites. The Rf value is computed by dividing the compound's distance from the starting point by the solvent front's distance (Bele and Khale, 2011) [2].

Methodology

Activation of Nanoparticles

Zinc oxide (ZnO) nanoparticles (NPs) from commercially available source were taken in a 50 ml centrifuge tube to which deionized water (5 ml) was added for washing (to remove the traces of impurities) and centrifuged. After centrifugation, NPs formed a pellet at the bottom of the tube and the deionized water added was discarded manually. To activate the NPs, Aminosilane was added and incubated for 1-6 h (@28 °C/150 rpm). After activation, activators are removed by subjecting to centrifugation and the supernatant was discarded followed by washing for 5 times with deionized water and two times with phosphate buffer solution in order to remove the traces of activators (Selvarajan *et al*., 2015) [12].

Immobilization of enzyme on activated ZnO NPs

β-Galactosidase was mixed with ZnO NPs and incubated with constant stirring in phosphate buffer (pH 6.5) at room temperature for 1-6 h. Samples were centrifuged and the enzyme immobilized NPs complex was washed thrice with 0.1 M phosphate buffer (pH 6.5) and twice with deionized water and finally stored in the same buffer at 4°C for further use (Selvarajan *et al*., 2015) [12].

Preparation of sample

The lactose solution of 40% was prepared by dissolving pure lactose in double distilled water. β-galactosidase enzyme was added in varying concentration i.e., 0.05, 0.1, 0.2, 0.4, and 0.6% to 100 ml lactose solution and solution were mixed at the speed of 100 rpm and temperature maintained at 40 °C. During hydrolysis, samples were collected at every 2 h interval till 8th hour followed by heating to 90 °C for 10 min to inactivate the enzyme.

Qualitative analysis of GOS by TLC

Analysis of GOS was carried out by using thin layer chromatography (TLC) method (Wang *et al*., 2021) [15]. The silica based commercially available TLC plates were purchased from Merck life sciences Pvt Ltd. A thin mark of about 2 cm was made at the bottom of plate with pencil to apply the sample spots with a concentration of 20 mg/ml for standards and hydrolysed samples, this plate was kept in TLC chamber for 1 h which is filled with a solvent or mobile phase (prepared using butan-1-ol, ethyl acetate, ethanol and water in the ratio of 3:1:1:1,v/v/v/v concentration). Maintain the sample spots well above the mobile phase. After 1h, the plate was removed from the chamber and dried completely. Further, it was sprayed with the developing agent i.e. 95:5 v/v concentration of ethanol and sulphuric acid (H_2SO_4) and dried in hot air oven at 110 °C for 10 min to visualize the spots. Retention factor (Rf) value was calculated after obtaining the spots on the plate using following equation:

$$\text{Retention factor } (Rf) = \frac{\text{Distance travelled by the component}}{\text{Distance travelled by the solvent}}$$

3. Results and Discussion

The results obtained from TLC has been shown in the Fig 3.1 respectively with different concentrations of enzyme.

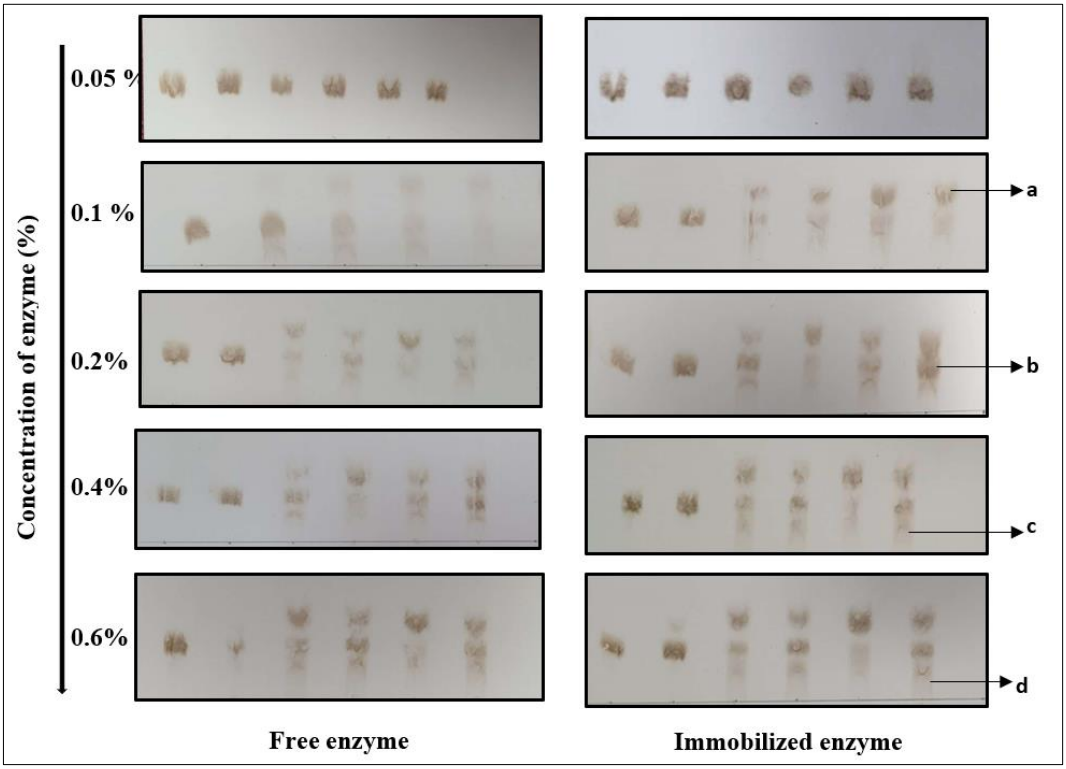

Fig 1: TLC plates showing the spots of Galactooligosaccharides at different enzyme concentration

[The first sample spot of the plate is for lactose standard and second is for 0th h hydrolysed sample similarly third, fourth, fifth and sixth is for 2nd h,4th h, 6th h and 8th h of hydrolysed sample. The region shown at point "a" is glucose, point "b" is for disaccharides, point "c" for raffinose and point "d" is for other triglycerides]

Qualitative analysis of GOS was determined through thin layer chromatographic technique in which mixture of components can be separated. The individual component present in it can be identified based on retention factor (Rf) of various components. The Rf value of various standards such as glucose, lactose and raffinose are determined (Fig. 4.6).

Based on the Rf values of standards, various components are identified from the sample spots are given in the Fig 4.5. At 0.05% concentration of enzyme, there was no production of GOS was observed. The production of GOS was observed above 0.1% enzyme concentration. With increase in concentration of enzyme, concentration of GOS was increased and samples treated with immobilized enzyme (at lower concentration) showed thicker GOS bands indicates higher efficiency of GOS production. Gupta et al. (2011) [8] reported that with increase in immobilization yield, the enzyme activity increased until optimum activity level is reached; further increase in immobilization yield decreased the enzyme activity because the surface of the nanoparticle becomes more crowded with so many protein molecules which lead to conformational deactivation. Similar results are also reported for pectinase immobilized on nanoparticles (Seenuvasan *et al*., 2014) [11], pectinase immobilized on magnetic chitosan particles (Dal *et al*., 2019) [3], α-amylase immobilized on magnetic nanoparticles (Dhavale *et al*., 2018) [4], β-galactosidase on silicon dioxide nanoparticles (Tizchang *et al*., 2020) [13], β-galactosidase on enzymatically active bacterial cellulose (Estevinho *et al*., 2018) [5] etc.

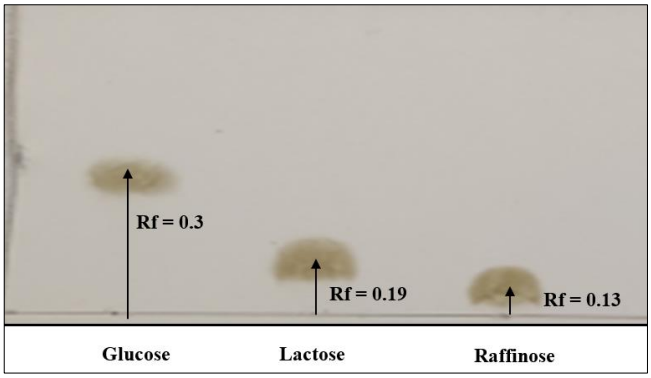

Fig 2: TLC plate depicting the spots of standards

Similarly, the production of GOS by *Lactobacillus fermentum* was confirmed by TLC (Mahadevaiah *et al*., 2020) [9]. The sample spots obtained on TLC plates were identical with the standard GOS and they reported that these GOS spots are combination of di, tri and tetra saccharides which further confirmed through HPLC. Aslan and Tanrıseven, (2007) [1] used both free and immobilized enzyme for GOS production and they found that product which is obtained from hydrolysis of free enzyme contained 68.2% lactose, 12.8% 6'-galactosyl lactose, 11.8% glucose and 7.2% galactose whereas product obtained from hydrolysis of immobilized enzyme contained 61.4% lactose, 15.8% 6'-galactosyl lactose, 14.2% glucose and 8.6% galactose. The higher efficiency in production of GOS through immobilized enzyme is due to hydrophobic character of immobilization matrix which allows lower water concentration for enzyme. They have also reported that there was no decrease in activity after using the immobilized enzyme in 20 batch reactions.

Conclusion

The present study was aimed to immobilize β-galactosidase on zinc oxide nanoparticles (ZnO NPs) and evaluate its effect on production of galactooligosaccharides at different enzyme concentration. The study revealed that above 0.1% concentration of enzyme GOS was observed on TLC plates, with increase in concentration of enzyme, concentration of GOS was increased and samples treated with immobilized enzyme (at lower concentration) showed thicker GOS bands which indicates higher efficiency of GOS production.

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