Original Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

APPLICATION OF SOLUBLE AND IMMOBILIZED α - GALACTOSIDASE FROM *ACINETOBACTER* SP FOR DEGRADATION OF GALACTO-OLIGOSACCHARIDES IN COWPEAS

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ABSTRACT

α-galactosidase purified from *Acinetobacter* sp. was immobilized in four different matrices- Calcium alginate, Gelatin, Agarose and *k*-Carrageenan. On comparative analysis, *k*-Carrageenan immobilized enzyme showed highest immobilization yield of 68% and storage stability for 30 days. Gelatin showed an immobilization yield of 57%. Soluble enzyme led to decrease of 84% in raffinose and 76% in stachyose concentration in cowpeas whereas *k*-Carrageenan immobilized enzyme led to decrease of 72% of raffinose and 54% of stachyose thereby decreasing the flatulence to greater extent.

KEYWORDS: ***k*-carrageenan, immobilization, galacto-oligosaccharides, α-galactosidase, cowpeas, flatulence**

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Received on: 05.11.2016
Revised and Accepted on: 17.1.2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.1.b596-602

INTRODUCTION

Cow peas or Black eyed beans (*Vigna unguiculata*) are rich source of dietary proteins, galacto-oligosaccharides, vitamins and minerals[1]. Abdominal discomfort after consumption of cow peas restricted their intake as food [2,3].This is due to the presence of galacto-oligosaccharides ie stachyose and raffinose that contributes to 50% of gas or flatulence [4, 5]. Absence of human α-galctosidase passes the undigested sugars to large intestine where they are digested and metabolised by intestinal microflora resulting in production n of gas [6]. Traditional methods like soaking and sprouting degrades the galacto-oligosaccharide but not to an extent of 100% [7]. Processing of legumes by using enzyme alpha galactosidase is one of the solutions to increase the nutritive value. Enzymatic treatment of cow peas flour with crude fungal α-galactosidase was reported to decrease concentration of galacto-oligosaccharides to a great extend [8, 9]. Immobilization technology has been successfully used in the bioprocess development for various applications, waste water treatment [10, 11, 12, 13, 14, 15] and enzymes production [16, 17, 18, 19]. Immobilization technology offers several advantages over free cell or enzyme systems. Particularly immobilization method helps the catalyst attach inert material thereby providing sufficient contact time for the substrate and complete the desired reaction. Also immobilization system reduces the amount of catalyst used (eg cells, enzymes) by remaining in process environment rather leaving the system when operated in continuous mode. This aids in lowered amount catalyst use there by efficient process in terms of resource utilisation. Industrially, both soluble and immobilized α-galactosidases are used in processing of soya milk [20, 21, 22, 23] and chick peas [24, 25] and cow peas. In the present study, enzyme α-galactosidase purified from *Acinetobacter* sp was applied enzymatically to degrade galacto-oligosaccharides and thereby increase the nutritive value of cowpeas [26, 27].

MATERIALS AND METHODS

Immobilization of alpha-galactosdiase enzyme

To verify the suitability of enzyme entrapped beads, purified alpha-galactosidase was immobilized in four different matrices.

Calcium Alginate immobilization

1ml of purified alpha-galactosidase (15.2U/ml) was mixed with 4 ml of sodium alginate to make a final concentration of 3%. The mixture was loaded in a syringe and extruded drop by drop into 0.2M $CaCl_2$ solution. The beads formed were allowed to harden in $CaCl_2$ for overnight. Later beads were washed in sterile water and stored in 20mM Tris buffer, pH 7.0 at 4°C for further use [28].

Gelatin immobilization

10% (v/v) sterile gelatin was prepared and maintained at 45°C. To this gelatin solution, 1 ml of purified Ag-I (15.2U/ml) was added, mixed well and poured into a sterile petridish. The gel was covered with 10 ml of 5% glutaraldehyde for solidification at 30°C. The gelatin blocks were cut into equivalent size cubes (5 mm^3) and the cubes were thoroughly washed with sterile distilled water until complete removal of excess glutaraldehyde [28].

k-Carrageenan immobilization

3% (w/v) K-carrageenan solution was dissolved in physiological saline (0.9NaCl). The solution was sterilized and cooled to the 40°C. To this solution 1 ml of purified Ag-I (15.2U/ml) was added, mixed well and poured into the petriplates. After solidification the *k*-Carrageenan blocks were sliced into equal size cubes (5 mm^3) and added to sterile 2% potassium chloride solution. The resulting cubes were treated for 3 min with 1% glutaraldehyde. After that the cubes were washed with sterle distilled water and stored in refrigerator at 4°C until use [28].

Agarose immobilization

4% of agarose solution was prepared with buffer solution. The solution was sterilized and cooled to 40°C. To this solution 2 ml of purified Ag-I (15.2U/ml) was added to make a final concentration of 2%. The solution was mixed gently without any foam and poured into the petriplates. After solidification, the agarose was sliced into equal size cubes (5 mm^3) and added to sterile Tris buffer (20mM, pH 7.0) and kept in the refrigerator (1 hour) for curing. After that the cubes were washed with sterile distilled water and stored in refrigerator at 4°C until use [28].

Reusability of the immobilized enzyme

Repeated batch experiments were done to establish the reusability of the immobilized enzyme using substrate ρ-nitrophenyl-α-D-galactopyranoside (ρNPGal). For every batch, 0.25gms of immobilized enzyme was incubated with substrate at 50°C for 10 minutes. After every batch, the reaction mixture was decanted under sterile conditions and the beads/cubes were washed with distilled water. Then a fresh reaction mixture was added to the tubes and the assay was continued for next cycle. Subsequent batches were run at predetermined time intervals.

Activity yield of the immobilized enzyme

Activity yield of immobilized alpha-galactosidase in four different matrices was calculated using the following equation.

$$Activity\ yield\ (\%) = \frac{activity\ of\ immobilized\ enzyme}{activity\ of\ soluble\ enzyme} \times 100$$

Storage stability of the immobilized enzyme

The immobilized alpha-galactosidase enzymes were stored in Tris buffer (pH 7.0) at 4°C. The stability of the immobilized enzyme was determined by measuring the residual activity of the enzyme at predetermined time interval of 5 days and consequently up to 40 days.

Increase of nutritive value of cowpeas

Extraction of oligosaccharides from cowpeas

Cowpeas collected from local market were milled to flour and sieved. 10g of sieved flour was taken and extracted with 100ml of 70% (v/v) ethanol. The sample was kept in a shaker at 36°C, 150 rpm for overnight. Insoluble residues were removed by centrifugation at 5000g for 10 minutes. The supernatant was collected and concentrated for further use [24].

Enzymatic treatment of Cowpeas (batch reaction)

Enzymatic hydrolysis of oligosaccharides in cowpeas was done by both soluble and immobilized enzyme. In batch reaction, 15 U/ml of soluble enzyme and 0.4g of immobilized enzyme were incubated with 1 ml of cowpea supernatant was incubated at 50°C at different time intervals of 2, 4, 6, 8 and 10 hours. The enzyme reaction was stopped by boiling the reaction mixture for 10 minutes followed by addition of 1ml of 0.3M barium hydroxide and 1ml of 0.1M of zinc sulphate [29].

TLC analysis

100 µl of unhydrolysed and enzyme hydrolysed cowpeas supernatant were loaded on silica gel TLC plate along with standards galactose, raffinose and stachyose. The TLC plate was developed with solvents-n-propanol: ethylacetate: water (6:1:3) and air dried [30]. The plate was sprayed with the spraying reagent (1% α-napthol in 95% ethanol containing 10% orthophosphoric acid) and the plate was air dried and kept in an oven at 100°C for 10-15 min. Sugar spots were identified based on retention factor.

Quantitative Analysis

The oligosaccharides were further quantitatively estimated by Tanaka et al. 1975 method. Corresponding oligosaccharides on TLC plate were scrapped and dissolved in 3ml of distilled water for 10 hours. The extract was filtered through whatman No.1 filter paper. To 1ml of filtrate, 1ml of 0.02M thiobarbituric acid and 1ml of conc. HCl were added. The reaction mixture was incubated for 10 minutes in a boiling water bath and later cooled. After cooling, the OD values were taken spectrophotmeterically at 430nm. The corresponding concentration was determined from a standard curve previously prepared from each reference sugar [31].

HPLC analysis

HPLC analysis was performed with a Shimadzu LC-20 with refractive index detector. Oligosaccharides are separated on a reverse phase C-18 column (250 x 4.6mm, 5mu). The mobile phase consisted of acetonitrile: ammonia (80:20). The flow rate was maintained at 1ml/minute. The peaks in untreated and enzyme treated samples were identified with retention time values of the standards

RESULTS AND DISCUSSION

Comparative analysis of immobilized enzyme

The purified alpha-galactosidase was immobilized in four different matrices. A comparative analysis of four different matrices was given table 1. Agarose showed lowest activity yield of 41%. The cubes of agarose were fragile and cannot be repeatedly used for conversion of substrate to product. Although sodium alginate beads were white, transparent, spherical and uniform in size, leakage of enzyme into substrate solution or reaction mixture was detected. *K*-carrageenan showed highest activity yield of 68% followed by gelatin 57%. The storage stability and reusability of both matrices- *K*-carrageenan and gelatin was almost similar. *K*-carrageenan immobilized enzyme was able to retain > 20% even after 10 repeated batch reactions. The stability of gelatin and *K*-carrageenan may be attributed to cross linking with glutaraldehyde solution. Alpha-galactosidase purified from *Aspergillus oryzae* was also reported to be less stable in calcium alginate compared to other matrices-gelatin and gelatin-alginate fibres [23].

Table 1
Comparison of immobilized alpha-galactosidase activity in four different matrices

Matrix	Activity of soluble enzyme U/g of beads	Activity of immobilized enzyme U/g of beads	Activity yield (%)	No. of cycles	Storage stability (days)
Sodium alginate (3%)	12.16	6.0	50	7	>25
Gelatin (10%)	13.6	7.74	57	11	30
Agarose (2%)	12.16	4.96	41	5	10
k-Carrageenan (3%)	12.92	8.84	68	11	30

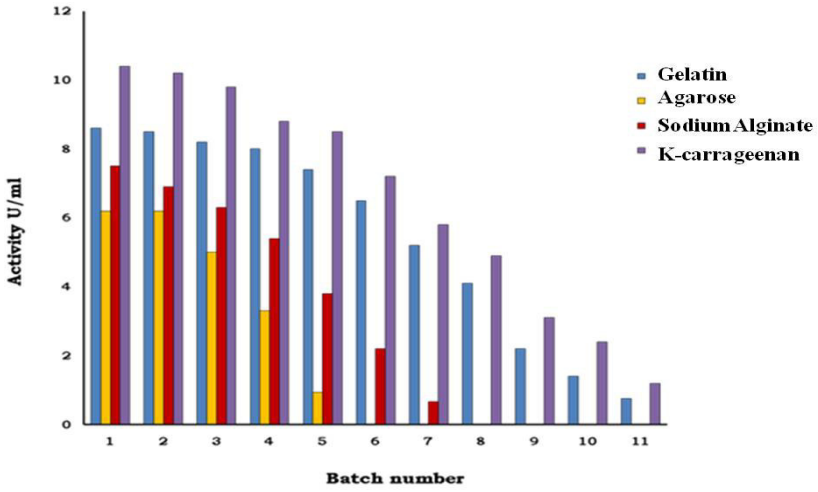

Figure 1
Reusability studies on four matrices-gelatin, agarose,sodium alginate and K-carrageenan

Figure 1 shows the reusability of four different matrices in repeated batch reaction. Gelatine immobilization sometimes may lead to denaturation of enzyme due to freezing and thawing procedure. Thus *K*-carrageenan which was more stable than other matrices was chosen for further experiments.

Enzymatic batch hydrolysis of cowpeas

Cowpeas were subjected to enzymatic hydrolysis of oligosaccharides by *k*-Carrageenan immobilized enzyme and soluble enzyme. Figure 2A and B shows the batch reaction hydrolysis of raffinose and stachyose at time intervals of 2, 4, 6, 8 and 10 hours. After 10 hours of incubation, oligosaccharide hydrolysis led to decrease of 84% in raffinose and 76% in stachyose concentration whereas immobilized enzyme led to decrease in 72% and 54% of raffinose and stachyose concentrations. Soluble enzyme showed higher efficiency of hydrolysis compared to immobilized enzyme.

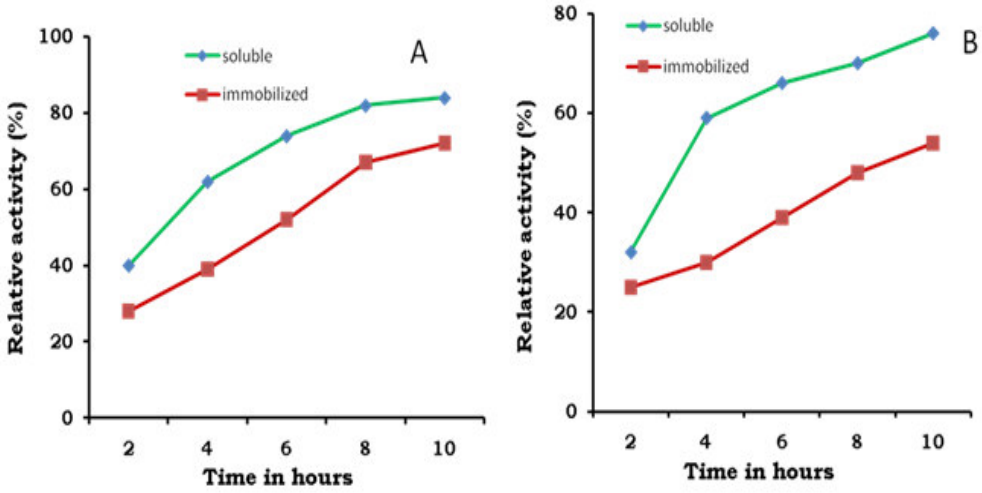

Figure 2
Enzymatic batch degradation of Raffinose
(A) and Stachyose (B) by both soluble and k- carrageenan immobilized enzyme

This may be attributed to the mass transfer resistance or limitation of immobilized enzyme to the substrate. It was also observed that as the time of incubation increased, rate of hydrolysis decreased. This may be due to substrate depletion or product inhibition. Richard and Esther, 1993 had reported the effect of soaking, cooking and crude alpha-galactosidase enzyme on oligosaccharide content of cowpeas. Soaking for 16 h resulted in an average reduction of 26·2% for stachyose and 28·0% for raffinose, while cooking for 50 min resulted in a reduction of 28·6% for stachyose and 44·0% for raffinose. Similar reports of hydrolysis of oligosaccharides in soya milk with soluble and *k*-Carrageenan immobilized alpha-galactosidase enzyme were reported by kotiguda et al.2007 [32].

TLC and HPLC analysis

TLC and HPLC analysis were carried out using galactose, raffinose and stachyose as standards (Figure 3 and Figure 4). On HPLC analysis, peaks for standards galactose, raffinose and stachyose were observed at retention time of 5.8, 10.5 and 17.1.

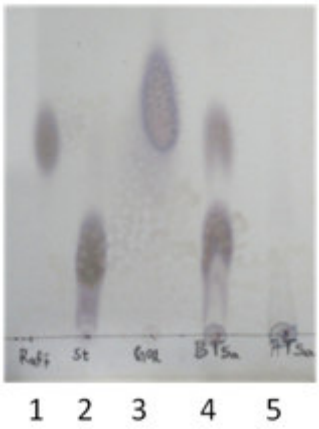

Figure 3

TLC Analysis of enzymatic galactooligosaccharide hydrolysis of cowpeas. Lane 1-Raffinose standard, Lane 2-Stachyose standard, Lane 3- Galactose standard, Lane 4-Unhydrolysed cowpeas sample and Lane 5-Enzymatically hydrolysed cowpeas sample

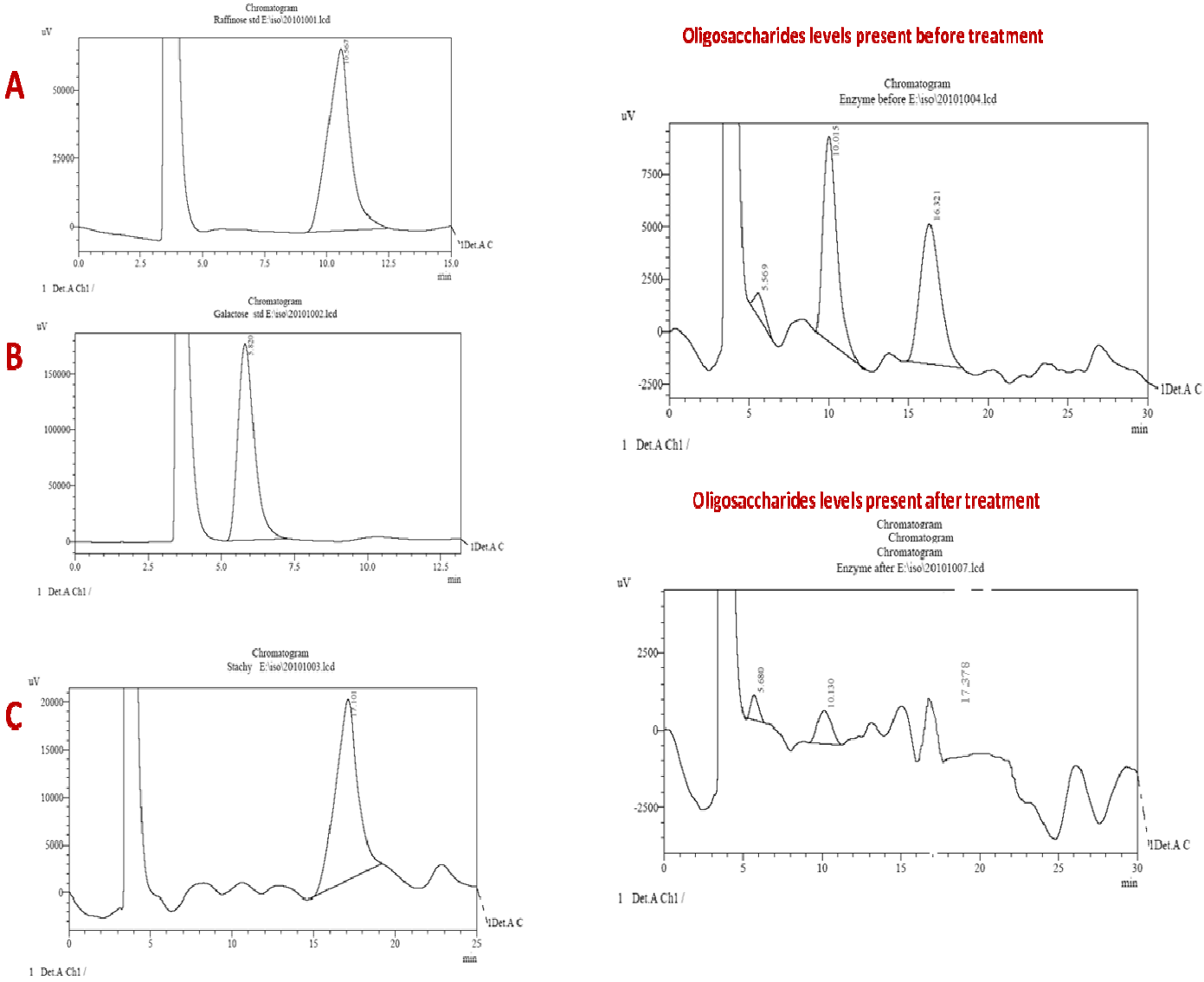

Figure 4

HPLC Analysis of enzymatic galactooligosaccharide hydrolysis of cowpeas. A- Raffinose, B-Galactose, C-Stachyose, D- Unhydrolysed sample and E- Enzymatically hydrolysed sample

Similar peaks corresponding to retention time of standards were observed before and after enzyme treatment. A decrease in the oligosaccharides (raffinose and stachyose) concentration after enzyme treatment was observed whereas there was no reduction in galactose concentration.

CONCLUSION

In present study, soluble and *k*-Carragennan immobilized enzyme showed a decrease in level of galacto-oligosaccharides in cowpeas. The study showed that raffinose hydrolysed is more in comparsion to stachyose under same optimal conditions. The results showed that the enzymatic processing proves to be more effective in decreasing the flatulence and increasing the nutritional value of cowpeas flour which is commonly referred as “poor man food”.

ACKNOWLEDGEMENT

The authors would like to thank Centre for Biotechnology, Institute of Science and Technology, JNTUH, Hyderabad for the support provided.

CONFLICT OF INTEREST

Conflict of interest declared none.

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