

## Production of Galacto-oligosaccharides by marine derived fungus *Aspergillus flavus*

A. PAVANI, T. PRABHAKAR and G. GIRIJA SANKAR

A. U. College of Pharmaceutical Sciences, Andhra University,  
Visakhapatnam - 530 003 (India).

(Received: November 02, 2008; Accepted: December 21, 2008)

### ABSTRACT

The aim of this research is to develop a cost effective method for the synthesis of oligosaccharides by using *Aspergillus flavus* (MTTC 9349) screened from marine fungus. This study was carried out, with a view of the marine strains may have better potential than their terrestrial counterparts. This is due to essential nutrients provided by marine biotopes for nurturing the isolates, and to extreme environmental niches. The marine fungi and their by-products may have potential values in food processing, fermentation, pharmaceutical and biopolymer industries. In this project we developed a production process of galacto-oligosaccharides (GOS), also known as Bifidus growth factor, are produced from lactose. The synthesis of GOS carried out by permeabilized and mutated cells of *A. flavus*. The process was optimized with different chemical agents for permeabilization; at substrate (lactose) concentrations ranging from 0.5 M to 2.25 M and pH.

**Key words:** *A. flavus*, Marine derived fungus,  $\beta$ -galactosidase, GOS. Bifidus factor.

### INTRODUCTION

The term "oligosaccharides" is broadly used for saccharides having the degree of polymerization of 2–10 (Nakakuki 1993). Structurally, Galacto oligosaccharides are composed of 2–20 monosaccharide residues linked by glycosidic bonds that are readily hydrolyzed to their constituent monosaccharide's either by acids (chemical) or by specific enzyme beta galactosidase (Prenosil *et al.*, 1987) (enzymatic). The enzymatic synthesis of GOS from lactose has been reported by many authors (Tzortzis, Goulas, Gibson, 2005; Onishi, & Tanaka 1998; Wierzbicki & Kosikowski 1972; Cruz *et al.*, 1999). The concentrations and structures of the oligosaccharides produced are mainly dependent on the concentration of substrate (Boon *et al.*, 2000). GOS stimulate the proliferation of lactic acid bacteria and bifidobacteria in the human intestine (Sako, Matsumoto, & Tanaka, 1999).

In this paper, a study on the enzymatic production of GOS from lactose by immobilization of whole cells of *A. flavus* was reported. The chemical synthesis of GOS requires multiple protection and de-protection steps (Sears & Wong, 2001). This complexity does not render this synthetic route attractive for industrial applications. To overcome this limitation, new strategies for the production of GOS by various methods of immobilizations were used (Aziz Tannriseven, Senay Dogan *et al.* 2002). Purification of enzyme is expensive process, so it is desired to develop a new inexpensive method of production of GOS by using whole cells. The crude enzyme also performed well (Barbara Splechtna *et al.* 2006) for the production of GOS to avoid expensive purification process. (Tzortzis, Goulas & Gibson, 2005) Whole cells of a novel strain, *Bifidobacterium bifidum* was used for the production of GOS.

Marine derived fungus *A. flavus* which is proven for the production of  $\beta$ -galactosidase, therefore it of interest to find whether the fungus *A. flavus* can produce GOS comparable to the GOS produced by the pure enzyme immobilization. In this study we try to find out the optimum conditions for the production of GOS from lactose by mutated cells of *A. flavus*.

### Mechanism of action

In the enzymatic hydrolysis of lactose, the enzyme  $\beta$ -galactosidase transfers a galactose moiety to an acceptor containing a hydroxyl group. If this acceptor is a water molecule, galactose is liberated (hydrolysis). When a sugar moiety acts as the acceptor, galactosyl-oligosaccharides (GOS) are formed. (Fig.1) In addition to hydrolysis,  $\beta$ -galactosidase from various micro organisms is also known to catalyze trans-galactolysation reactions. The formed galactosyl-oligosaccharides vary in chain length and in the inter connection of the monomer units (Crittenden & Playne1996).

GOS are usually considered as non-digestible, mildly sweet, poorly viscous, water-soluble sugars. They are functional food ingredients with a great potential to improve the quality of many foods. GOS have various physiological functions, such as the improvement of the intestinal microflora based on the selective proliferation of *Bifidobacteria* and the stimulation of mineral absorption.

## MATERIAL AND METHODS

### Chemicals

Lactose monohydrate was supplied from Merck; 2-nitrophenyl-  $\beta$ -D-galactopyranoside was procured from Carbosynth Limited, Berkshire, U.K. All other chemicals and medium constituents in this study were of analytical grade and procured from Sigma-Aldrich and s.d fine chemicals.

### Microorganism

The *Aspergillus flavus* derived from marine fungus collected at Bay of Bengal near Vishakapatnam in August 2005, using dilution-plate method (Christensen 1963) on PDA medium (Nielsen & Sorensen 1997). It was identified according to its morphological characteristics and confirmed by Prof. Ananthpadnabhan (IMTECH,

Chandigarh). Working stocks were prepared on Potato Dextrose Agar slants and stored at 4° C. *A. flavus* was immobilized in calcium alginate and used for the production of GOS.

### Enzymatic synthesis of GOS

Lactose solutions were prepared in different concentrations ranging from 0.5 M to 2.25 M in 50 mM potassium phosphate buffer containing 1 mM MgCl<sub>2</sub>. Different reaction conditions were assayed: temperature (25°, 37°, 40°, and 50°C), pH (4.5, 5.5, 6.5, 7.0, 7.5, 8.0), Immobilized *Aspergillus flavus* cells 2%. Experiments were carried out in duplicate. Reactions were performed in EM flasks incubated in an orbital shaker at 200 rpm. Samples were withdrawn at specific time intervals (30, 60, 120, 180, 240, and 300 min) and immediately immersed in boiling water for 5 min to inactivate the enzyme. The samples were stored at 4°C for subsequent analysis.

### Chromatographic determination of carbohydrates

The samples were analyzed by HPLC using amino column (Shodex Asahipak NH2P-50 4E) at room temperature. The column was eluted with acetonitrile : water (80 : 20) at a flow rate of 1 ml/min. The peaks were detected by refractive index detector and it was assumed that the response was independent on the degree of polymerization. The amount of each saccharide was calculated from the area of corresponding peak. The number of sugar units of the oligosaccharides was found on the basis of retention time, trisaccharides and tetrasaccharides were identified on the basis of having a retention time later than of disaccharides. Lactose, glucose, galactose, and higher saccharides, such as tri- and tetrasaccharides were measured as weight percentage.

Samples and standard solutions of glucose, galactose, and lactose were filtered through a Millipore (0.45 micron) membrane before injection in to HPLC column. Twenty micro liters were injected using an auto sampler and separations were performed at a flow rate of 1 ml/min. Quantification of each sugar was performed by external calibration using standard solutions of galactose, glucose, lactose. The yield of GOS was expressed as percentage of carbohydrate weight formed per

weight of lactose initially present. The peaks were measured with refractive index detector with the sample retention time 30 minutes.

found to be best permeabilizing agents for *A. flavus* followed by acetone.

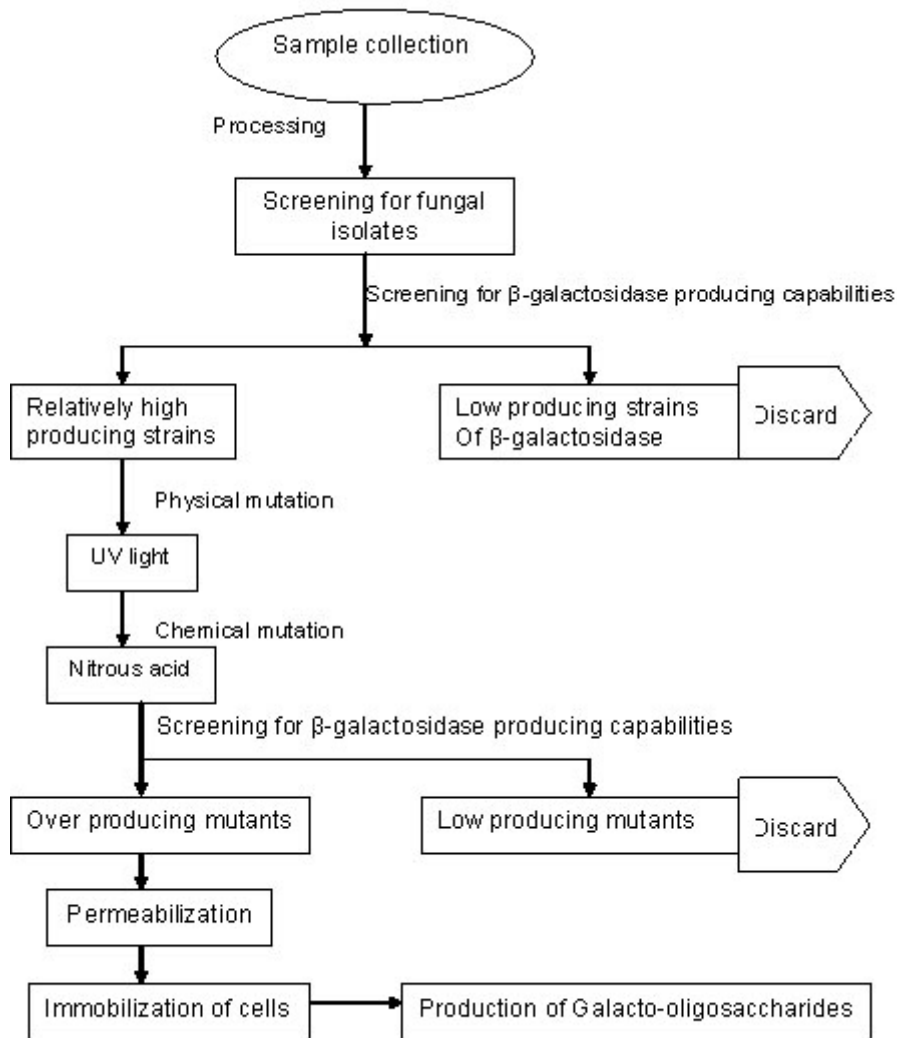
**RESULTS AND DISCUSSIONS**

**Permeabilization of *A. flavus* with different chemical agents**

Major part of enzyme  $\beta$ -galactosidase in cells of *A. flavus* was intracellular. So treatment with different chemical agent leads to permeabilization of cell wall there by the enzyme  $\beta$ -galactosidase, accessible with the substrate. Among various chemical agents (Fig 1.1) ethanol and toluene were

**Permeabilization with Detergents:**

Among different detergents of cationic, nonionic, anionic were tried. (Fig 1.2) Out of which Cetyltrimethyl ammonium bromide shows best permeabilizing activity of the mutated cells of *A. flavus*. Various concentrations of CTAB ranging from  $1.37 \times 10^{-3}$  M to  $1.09 \times 10^{-2}$  M concentrations were tried. The cells of *A. flavus* after treatment with CTAB of  $2.75 \times 10^{-3}$  M concentration (Fig 1.3) yields better  $\beta$ -galactosidase activity compare with the other organic solvents and detergents.



1.2. Flow diagram for the production of Galacto-oligosaccharides

(Gal: galactose; Glu: glucose; n = 1-7)

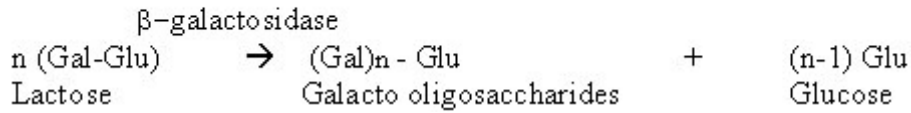


Fig. 1 Enzymatic reaction of lactose to Galacto oligosaccharides and glucose

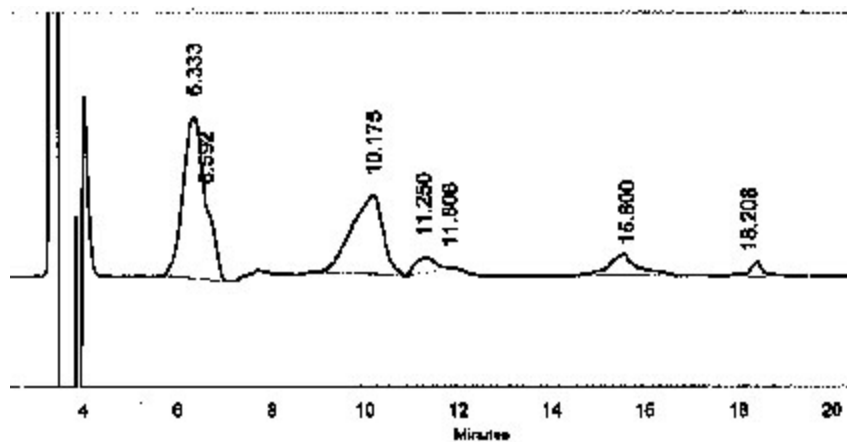


Fig. 1.0: HPLC chromatogram: carbohydrate profile obtained from lactose hydrolysis produced by immobilization of *A. flavus* at pH 7.00, 40° C, 2.25 M of lactose solution. The identified compounds are indicated: (1) galactose, (2) glucose, (3) lactose, and (4, 5 and 6) Galacto oligo saccharides

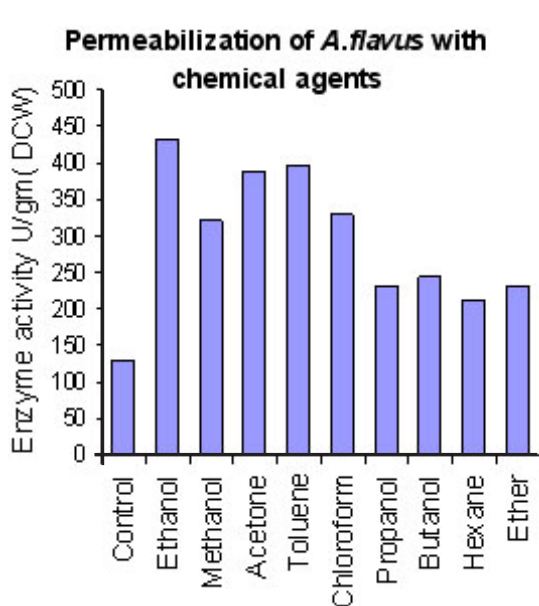


Fig. 1:1

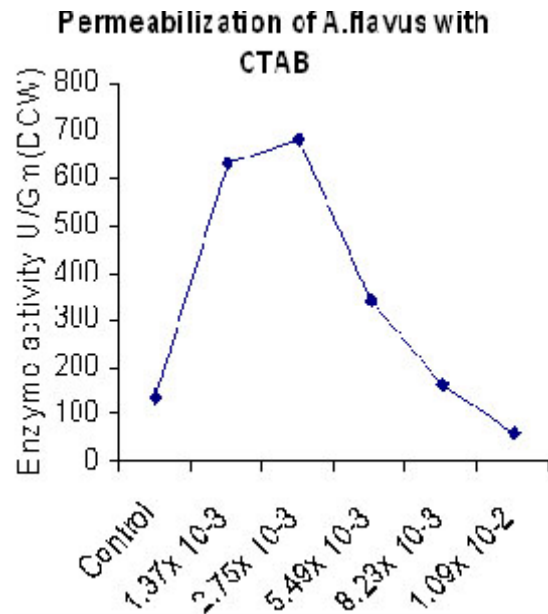


Fig. 1:2

**Effect of substrate concentration on production of GOS**

Mutated and permeabilized cells of *A. flavus* were immobilized in sodium alginate and used for the production of GOS. For initial lactose concentrations of 0.5M, 0.75M, 1M, 1.25M, 1.5M, 1.75M, 2M and 2.25M were tried and the maximum GOS yields at 37°C were found to be 4.2 %, 6.4 %,

12.4 %, 16.7 %, 22.1%, 23.9 % after 160 minutes, respectively. Transgalactosylation significantly increased with the increased lactose concentrations (Fig 1.4). This is due to the fact that, at a low lactose concentration, hydrolysis is favoured since the amount of hydroxyl groups of carbohydrates is lower as compared to those of water, which act as acceptors of galactose.

**Table 1: Effect of pH on the production of GOS**

% of saccharides	pH 4.5	pH 5.5	pH6.5	pH 7.0	pH7.5	pH 8.00
Glucose	16.2	39.4	41.6	35.2	33.2	21.6
Galactose	3.6	9.2	9.4	9.1	10.1	5.8
Lactose	70.7	39	32.5	34.9	40.4	59.3
GOS	9.4	12.3	16.6	20.8	16.3	13.3

**Effect of pH on the production of GOS**

We also investigated the effect of pH on the production of GOS by using wide range Mcilvaine buffers at pH 4.5 to 8.0 at lactose concentrations 1.75M. The decrease in the lactose concentration is rapid due to hydrolysis of lactose at pH 5.5, but the amount of GOS formed was higher at pH 7.00. (Table 1.0). β-galactosidase from either *E. coli* or *S. cerevisiae* reported that optimum pH for production of oligosaccharides were little

higher than optimum pH for hydrolysis (Hubber et al., 1976). Our findings are consistent with the reported data.

**Reusability of immobilized A. flavus cells**

Cells were reused up to 8 times for subsequent synthesis reactions with equal amounts of GOS being formed at analogous periods of time. From this point onwards, and always at the same reaction times, a slight decrease on the produced

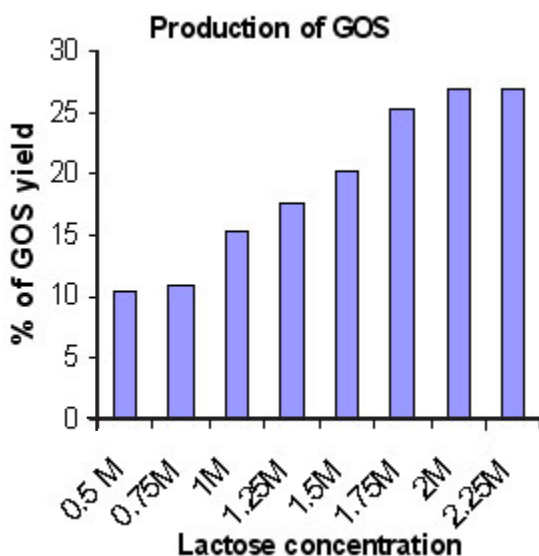


Fig. 1:3

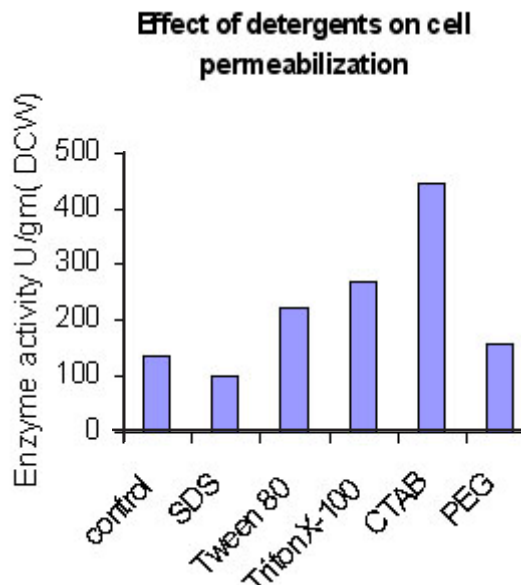


Fig. 1:3

oligosaccharides was observed (compared with the initial eight reactions), resulting to a total 10% decrease in the synthesis of oligosaccharides after 12 times of re-use.

Lactose solution (1.75 M) at different pH was incubated at 37°C with mutated cells of *A. flavus* at a concentration of 2% for 360 minutes.

Our results show that immobilization of whole cells was cost effective for the production of GOS. But the yield of GOS was comparatively low, with the immobilization of *A. flavus*. Immobilization of semi purified enzyme or crude extract also performed well in the production of GOS from lactose (Barbara Splechtna *et al* 2007). Degree of lactose conversion to GOS, mainly depend on the initial lactose concentration, the buffer (pH), salt concentration, temperature.

## CONCLUSION

Based on the results reported in this study,  $\beta$ -galactosidase from *A. flavus* can be considered for the GOS production. To reduce enzyme costs, we successfully used the permeabilized *A. flavus* cells for the production GOS, thereby avoiding expensive purification of the enzyme. Moreover, the *A. flavus* cells can survive at 40°C in immobilized form causes, improved yield of GOS and decrease in microbial contamination.

## ACKNOWLEDGEMENTS

The author is very thankful to Dr R.S. Prakasham, Indian Institute of Chemical Technology Hyderabad for providing necessary facilities for analysis of Galacto oligosaccharides and his valuable suggestions.

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