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# OLIGOSACCHARIDES SYNTHESIS BY FREE AND IMMOBILIZED ß-GALACTOSIDASES FROM *THERMUS AQUATICUS* YT-1.

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**SUMMARY.** Galactooligosaccharides (GalOS) production from a lactose solution (16% w/v) was studied at 70°C, pH 4.6 and 6.0 with free or immobilized ß-galactosidases of *Thermus aquaticus* YT-1. Synthesis of OS and lactose conversion was significantly improved when using the immobilized preparation. At pH 4.6, OS accounted for approx. 35% of the total carbohydrates with a conversion rate of lactose of 80%. The general formula of OS synthesized in this way was Gal-(Gal)n-Glu with n being equal to 1 or 2 and a ß anomeric configuration of the D-galactosyl moiety.

## INTRODUCTION

Although there are numerous advantages to operate processes at high temperature, production and use of enzymes from thermophilic microorganisms in the food industry are not well developed. The real potential of such microorganisms and enzymes is likely to be in new applications rather than to replace current biotechnological process (Zamost *et al.*, 1991). Only few oligosaccharides have been synthesized at high temperature. Synthesis of some alkyl ß-D-glycosides by transglycosylation using a crude extract of *Sulfolobus solfataricus* (Trincone *et al.*, 1991), the production of cyclodextrins by cyclodextrin-glycosyl transferase (CGTase) of *Bacillus stearothermophilus* (Shibuya *et al.*, 1993) and galactooligosaccharides (GalOS) by galactosyl transferase activity of *Thermus aquaticus* YT-1 (Berger *et al.*, 1995a) have been reported. Interest in the GalOS is growing because these products have been shown to promote the growth of Bifidobacteria (Ito *et al.*, 1990). Various parameters such as source of enzyme, substrate concentration, pH, temperature, etc. can displace the equilibrium of the reaction catalyzed by ß-galactosidase towards the galactosyl transferase reaction to synthesize the desired OS (Zarate and Lopez-Leiva, 1990; Prenosil *et al.*, 1987). The state of the enzyme (free vs immobilized) is known to affect OS formation, but data in the literature are sometimes contradictory. Lower production of OS was observed after immobilization of ß-galactosidases of *Bacillus sp.* (Nakanishi *et al.*, 1983; Rugh, 1982). In contrast, increased trisaccharide synthesis by glutaraldehyde treated or immobilized ß-galactosidases from *B. circulans* as compared with free enzyme was reported (Mozaffar *et al.*, 1989, 1987).

In a previous study, crude extract with galactosyl transferase activity of *T. aquaticus* YT-1 was immobilized with high yield into agarose beads using a co-crosslinking and entrapment procedure (Berger *et al.*, 1995b). The reaction equilibrium was shifted towards OS synthesis using high concentration of substrate and viscosity was reduced as a result of the high temperature of the process. Two types of OS were synthesized from lactose at 70°C either in acidic or neutral conditions.

The objectives of this study were (i) to study the effect of immobilization on OS production either at pH 4.6 or 6.0 in a batch process and (ii) to determine the partial composition of OS synthesized by ß-galactosidases from *T. aquaticus* YT-1 from lactose at 70°C.

## MATERIALS AND METHODS

**Strain and preparation of the enzyme solution.** *Thermus aquaticus* YT-1 (ATCC 25104) was from the American Type Collection Culture (ATCC, Rockville, MD, USA). YT-1 cells were grown in 0.1% TYE Castenholz medium pH 7.6 (Medium 461, ATCC Media Handbook, 1984) containing 1% filter-sterilized cellobiose at 70°C. The crude cell-free extracts were prepared as previously described (Berger *et al.*, 1995b).

**Immobilization technique.** The enzyme solution was immobilized by co-crosslinking with bovine serum albumin followed by entrapment in agarose beads (Berger *et al.*, 1995b). Beads of approx. 2.0 mm were formed and stored until use at 4°C in McIlvaine buffer pH 6.0 or pH 4.6. This immobilized enzyme preparation (IE) was used for OS synthesis.

**Oligosaccharide synthesis.** GalOS synthesis and lactose hydrolysis were studied at pH 4.6 and 6.0 at 70°C with the free enzyme (FE) or the immobilized enzyme preparation (IE). Synthesis with IE were done in 100 ml of 16% lactose solution (w/v) with a bead volume ratio of 40%. A similar enzyme activity was used to perform the synthesis in the different systems. Spinner Flask $\mu$-Carrier (Bellco Glass Inc., Vineland, N.J.) and Magna Flex™ (Wheaton, Millville, N.J.) reactors were used for FE and IE, respectively. Agitation (60 rpm) was performed using a 5 module magnetic stirrer (Type 45700 Cellgro™ Stirrer, Thermolyne Sybron, Dubuque, IA.). The reaction mixture was overlaid with mineral oil to prevent evaporation during sampling. The reaction was stopped by addition of 1.2 M cold $H_2SO_4$. Samples were centrifuged, filtered through a 0.22 $\mu$m membrane and analyzed by HPLC. Experiments were performed in duplicate.

**High Performance Liquid Chromatography.** Hydrolysis and transferase products were monitored using a Bio-Rad HPLC system (Bio-Rad Laboratories Ltd., Mississauga, Ont., Canada). Analyses were performed isocratically on a 300 mm x 6.5 mm Sugar Pak I column (Millipore Co., Waters Chromatography, Ville St-Laurent, Qc., Canada). The flow rate was 0.5 $ml.min^{-1}$ at 85°C with $10^{-4}$ M EDTA as mobile phase.

**Purification and partial elucidation of oligosaccharides.** Oligosaccharides were separated from monosaccharides on an activated charcoal column equilibrated with water. OS were eluted with a step gradient of ethanol and concentrated in a rotatory evaporator. The resulting syrup was further purified by injections through two Sugar-Pak I columns in series as described above.

The molar carbohydrate composition of each recovered fraction was determined after acid hydrolysis with 0.5 M HCl for 4 h at 100°C and residues were analyzed by HPLC. The anomeric configuration of the D-galactosyl group was determined using ß-galactosidase from *Escherichia coli* (Boerhinger Mannheim, Laval, Qc., Canada) and $\alpha$-galactosidase from coffee bean (Boerhinger Mannheim). The enzymatic hydrolysis for ß- and $\alpha$-galactosidases was performed in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM $MgSO_4$, 0.2 mM $MnSO_4$, 50 mM 2-mercaptoethanol at 37°C and in 0.1 M sodium phosphate buffer (pH 7.0) at 23°C, respectively. Hydrolysis products were analyzed by HPLC.

**Stastistical analysis.** Analysis of variance was carried out to determine significant differences among treatments using the general linear procedure GLM of SYSTAT© (SYSTAT©, 1990).

## RESULTS AND DISCUSSION

A series of batch experiments was performed to evaluate the synthesis of OS from a concentrated lactose solution by free and immobilized ß-galactosidases of *T. aquaticus* YT-1 at 70°C (Fig. 1). The galactosyl transferase activity of these ß-galactosidases with lactose leads to the synthesis of two products OS-1 and OS-2 in addition to the hydrolysis end-products glucose and galactose (Berger *et al.*, 1995a, 1995b). The concentration of glucose in lactose hydrolysate was higher than galactose (Fig. 1), which indicates that the oligosaccharides (OS) contain galactose moieties. This observation was confirmed by the structural composition of OS-1 and OS-2 which were established to be a tetrasaccharide (gal/glu 3:1) and a trisaccharide (gal/glu 2:1), respectively. Thus, the missing galactose corresponds to the additional moiety involved in the extention of the lactose molecule by the transgalactosylation reaction leading to the OS formation. The anomeric configuration of the additional galactose moiety is likely to be catalyzed under a ß anomeric linkage to the non-reducing end residue of the sugar. Complete structural elucidation of these OS is under investigation. Maximum OS synthesis was reached after approx. 24 h. The formation of OS appeared to follow an exponential curve, especially for the trisaccharide OS-2, but a plateau in the OS curves was observed for long reaction times. This is different from what was reported for ß-galactosidases of mesophilic

microorganisms, where the OS eventually decreased as a result of hydrolysis (Zarate and Lopez-Leiva, 1990; Mozaffar *et al.*, 1988; Prenosil *et al.*, 1987). In the different experiments, OS yield varied from 32 to 35% of the total carbohydrate concentration (Table 1). The highest OS synthesis was observed with the immobilized enzyme preparation in acidic condition ($P < 0.05$). The OS-2 production accounted for over 30% of total carbohydrates, while OS-1 represented only approx. 5%. Use of immobilized ß-galactosidases resulted in an increase of OS formation without affecting the type of OS.

The immobilized enzyme produced less OS-1 and more OS-2, compared to the free enzyme ($p < 0.001$). Because OS-2 (trisaccharide) is a precursor to the synthesis of OS-1 (tetrasaccharide), the reduced amount of OS-1 obtained with IE could be explained by the microenvironment inside the gel matrix. Lactose conversion was significantly higher with IE than FE ($P < 0.001$). About 80% conversion rate was achieved with IE at pH 4.6 as compared to 70% for free enzyme.

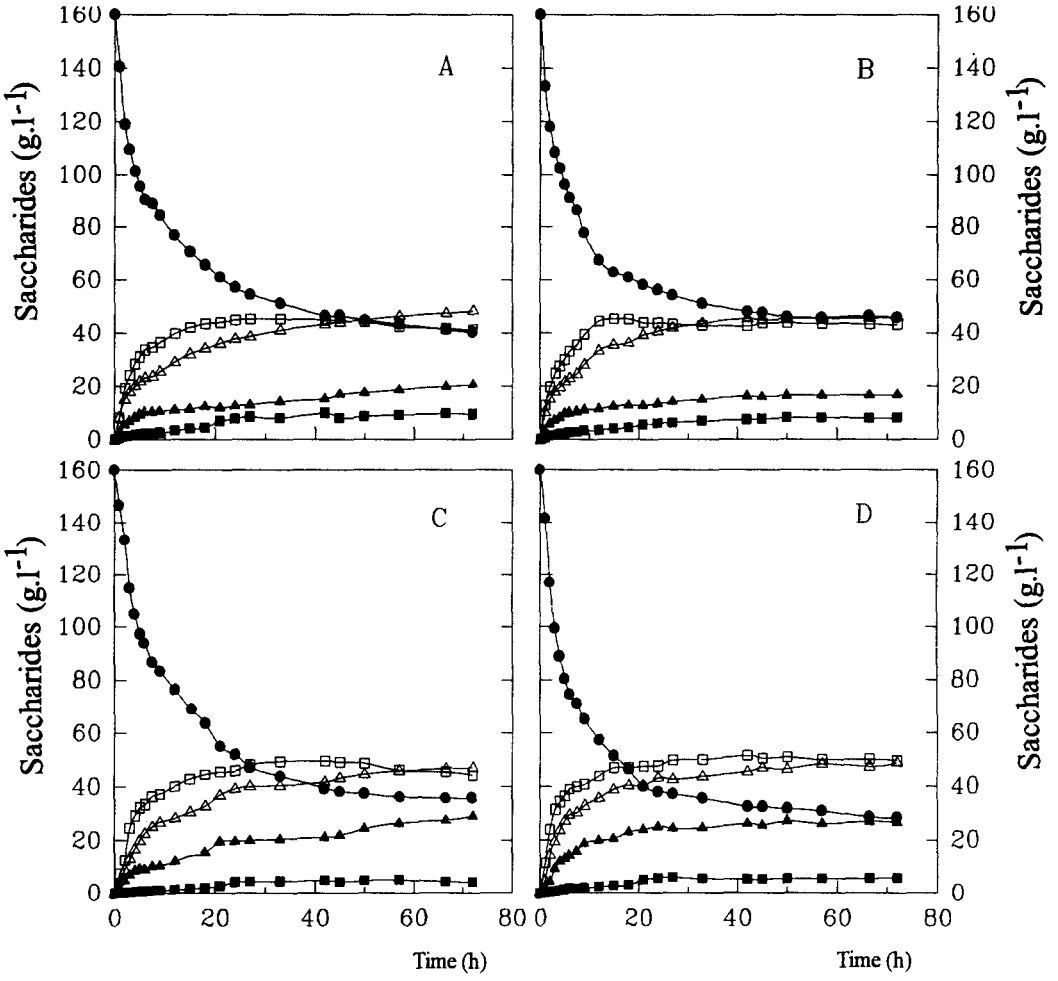


**Fig. 1** Kinetics of galactooligosaccharides (GalOS) synthesis from lactose (16% w/v) at 70°C by free (FE) and immobilized enzymes (IE) of *T. aquaticus* YT-1. Experimental conditions: **A:** FE, pH 6.0; **B:** FE pH 4.6; **C:** IE, pH 6.0; **D:** IE, pH 4.6. (●) Lactose; (■) OS-1 and (□) OS-2; (▲) galactose; (△) glucose.

**Table 1** Galactooligosaccharides (GalOS) synthesis with free and immobilized ß-galactosidases from *T. aquaticus* YT-1 in a 16% (w/v) lactose solution (pH 4.6 or 6.0) at 70°C.

| Experimental conditions. | Sugars[1] | Concentration[2] ($g.l^{-1}$) | Total sugar[2,3] (%) |
|---|---|---|---|
| Free enzyme pH 6.0 | OS-1 | 8.62 ± 0.30 | 5.50 ± 0.16 |
| | OS-2 | 42.32 ± 0.83 | 27.29 ± 0.26 |
| | Total OS | 50.9 ± 1.08 | 32.8 ± 0.44 |
| | Lactose | 43.78 ± 1.08 | 27.37 ± 0.68 |
| Free enzyme pH 4.6 | OS-1 | 7.47 ± 0.28 | 5.05 ± 0.06 |
| | OS-2 | 43.49 ± 0.38 | 27.37 ± 0.15 |
| | Total OS | 51.0 ± 0.62 | 32.4 ± 0.20 |
| | Lactose | 46.37 ± 0.33 | 29.16 ± 0.25 |
| Immobilized enzyme pH 6.0 | OS-1 | 4.59 ± 0.10 | 2.87 ± 0.06 |
| | OS-2 | 47.78 ± 0.79 | 29.86 ± 0.50 |
| | Total OS | 52.4 ± 0.82 | 32.7 ± 0.52 |
| | Lactose | 37.04 ± 0.65 | 23.77 ± 0.71 |
| Immobilized Enzyme pH 4.6 | OS-1 | 5.63 ± 0.08 | 3.52 ± 0.05 |
| | OS-2 | 50.01 ± 0.35 | 31.26 ± 0.22 |
| | Total OS | 55.6 ± 0.30 | 34.8 ± 0.19 |
| | Lactose | 30.81 ± 0.74 | 19.68 ± 0.57 |

[1] GalOS: OS-1, tetrasaccharide and OS-2, trisaccharide; residual lactose. Synthesis time: 72h.
[2] mean ± s. d.
[3] percentage of total carbohydrates.

Due to the high temperature of this enzymatic process, browning of the solutions was particularly observed at pH 6.0, either with IE or FE. At pH 4.6, the browning reaction was less pronounced. Discoloration and refining of the final product was achieved by using activated charcoal. In spite of the fact that the process was not performed in sterile conditions, no microbial contamination was observed. No physical or enzymatic degradation of agarose beads was noticed during the batch process.

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