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Factors affecting the production of lactulose by *Lactobacillus acidophilus* NRRL 4495 β-galactosidase and its biological activity

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ABSTRACT

Aim: Production of lactulose and other oligosaccharides by *Lactobacillus acidophilus* NRRL 4495 β-galactosidase and their biological activity.
Methodology and Results: The transgalactosylation activity of *Lactobacillus acidophilus* NRRL 4495 β-galactosidase was investigated under different conditions for synthesis of lactulose and oligosaccharides. The synthesis was optimized with respect to pH; time; enzyme concentration and substrates ratio (lactose: fructose). Maximum production for lactulose was found to be 25 g/L at pH 6.6 with 40: 20% (w/v) lactose to fructose, respectively and enzyme concentration 4 IU/mL after 7 h. With respect to the other oligosaccharides the maximum yield (19 .68 g/L) was obtained under the same conditions but with enzyme concentration 2 IU/mL and after 10 h. As a new pharmaceutical application the produced lactulose and oligosaccharide and their sulfated derivative were found to have fibrinolytic activity, but they failed to act as anticoagulant.
Conclusion significance and impact of study: the research leads to increase the production of lactulose and other oligosaccharides with a significant yield and discovered a new pharmaceutical application for all the products.

Key words: β-galactosidase, lactulose, oligosaccharides, anticoagulant activity, fibrinolatic activity.

INTRODUCTION

Prebiotic are defined as selectively fermented ingredients that allow specific changes, both in composition and/or the activity in the gastrointestinal microbiota, that confer benefits upon the host's well-being and health (Gibson, *et al*., 2004). To date, the only inulin, fructooligosaccharides (FOS), lactulose and galactooligosacvcharides (GOS) are considered as established prebiotics (Gibson, *et al*., 2004; Olano and Corozo, 2009). GOS are oligomers of galactose linked to terminal end of glucose or galactose. A number of positive health effects are associated with their consumption. These vary, and can include: enhancing calcium and magnesium absorption (Chonan *et al*., 1996; Van den Heuvel *et al*., 2000); assisting in the inhibition of the attachment of pathogenic bacteria to the colonic epithelium (Sinclair *et al*., 2009; Tzortzis *et al*., 2005). They also having potential as a therapeutic agent in irritable bowel syndrome (IBS) (Silk *et al*., 2009); preventing the incidence and symptoms of traveler,s diarrhea (drakoularakou *et al*., 2010) and stimulating the immune system (Vulevic *et al*., 2008). Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is currently produced by chemical synthesis involving the alkaline isomerization of lactose (Mendez and Olano, 1979; Parmjit and Shweta, 2011). Howerver, this method has several drawbacks, such as high level of lactulose degradation and a considerable amount of colored by-products that are difficult to separate. The required separation and purification steps to remove these by-products are costly and lead to low lactulose yields (Deya and Takahashi, 1991; Zokaee et al., 2002). Bioproduction of lactulose by enzymes is therefore adesirable strategy to overcome the disadvantage of lactulose production by chemical synthesis. β-galactosidase have been used in the production of galactooligosachharides from lactose via transgalactosylation reaction (Hung and Lee, 2002 Carlos *et al*.,2012). The occurrence of transgalactosylation reaction with β-galactosidase suggests the possibility for bioconversion of lactose and fructose into lactulose. The rate of transferase and hydrolyase activities of the enzyme affects the amount and nature of the formed oligosaccharide. Since the enzyme source; the concentration and the nature of the substrate and the reaction conditions (pH; temperature and time) are the main affecting factors (Carlos *et al*.,2011; Gaur, *et al*.,

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2006; Kim, *et al*., 2004). As it is known, disaccharides such as lactulose possesses an important prebiotic character (TUohy, *et al*., 2002; Parmjit and Shweta, 2011), therefore it is necessary to gain more insight on the formation not only of trisacchariedes but also on the disaccharide fraction during transgalactosylation reaction. The present study deals with factors (time; pH; enzyme and substrate concentrations) affecting the formation of the main disaccharides during lactose hydrolysis using β-galactosidase from *Lactobacillus acidophilius* NRRL 4495.

MATERIALS AND METHODS

Microorganism

Lactobacillus acidophilius NRRL 4495 was obtained from American type culture collection, USA. The culture was maintained on potato dextrose agar slant medium and after incubation at 30 °C for 48 h, stored at 4 °C perior.

Chemicals

Heparin, purchased from Sigma chemicals co., USA. Hemoclar, preperd by Clin-midy, Paris and purchased from Nil Co. pharmaceuticals, Cairo, Egypt. Plasma, was purchased from Egyvac-Vacsera company. All other chemicals were analytical grade.

Production of β-galactosidase

Inoculum culture was prepared according to Onishi, *et al*., 1995. *Lactobacillus acidophilius* NRRL 4495 was growing in a medium composed of (g/L): Whey 57 (equal to 35 g lactose); ammonium sulfate 30; K_2HSO_4 30; KH_2SO_4 10; $MgSO_4.7H_2O$ 50 (Siham, *et al*., 2010). The pH of the medium was adjusted to 7 before sterilization. Whey was sterilized separately. Fermentation was performed in 250 mL Erlenmeyer flask each contained 50 mL of the sterilized medium at a rotary shaker (180 rpm) at 40 °C for 24 h. The bacterial cell were harvested by centrifugation at 10.000 X g for 10 min at 4 °C and washed twice with 0.1 M sodium phosphate buffer (pH 6.8). The wet cells were ground in the same buffer with sterile sea sand in quartz mortar under cooling conditions. The extraction mixtures were centrifuged as indicated above and the supernatant was used as enzyme source in all the next experiments.

Enzyme assay

β-galactosidase activity was assayed according to the method described by Siham, *et al*., (2010).

Optimization of reaction conditions for lactulose production

To specify the suitable reaction conditions for lactulose production, different times; different pHs; different enzyme concentrations and different substrates ratio were tested. All the reactions were carried out at 40 °C. At the end of each reaction lactulose concentration was determined spectrophotometrically as described below.

Effect of reaction time

The optimal time for lactulose production was investigated in the reaction mixtures contain (per mL) 40% w/v lactose; 20% w/v fructose and 2 U enzyme at pH 7.0. At different times interval (from 3 to 24 h) samples withdrawn boiled for 5 min in water bath and tested for oligosaccharide as indicated below.

Effect of enzyme concentration

The optimal enzyme concentration was determined by using enzyme concentration varying from 2-10 U/mL of reaction mixtures of 40% lactose and 20% fructose at pH 7.0 for 7 and 10 h.

Effect of different pHs

The buffer systems of 0.2 M citrate-buffer (pH 5.5-6.5) and 0.2 M phosphate-buffer (pH 6.5-8) were used to test the effect of different pHs on the production of lactulose. The reaction mixtures of 40% lactose and 20% fructose and the most suitable enzyme concentration and at optimal time were allowed to proceed at different pHs.

Effect of different substrates ratio

The optimal ratio of lactose to fructose was determined with 60% (w/v) total sugar by varying the ratio of lactose to fructose as follows: 20:40; 40:20; 45:15; 50:10 and 30:30 respectively. The mixture from 20% galactose; 20% fructose and 20% lactose was also tested. Each reaction contained 8 U enzyme concentration in 2 mL reaction mixture at pH 7 and incubated at 40 °C for 7 h.

Identifiction and purification of lactulose and other oligosaccharides

Thin layer chromatography (TLC)

Lactulose and other oligosaccharides were separated by TLC using propanol: water (8.5: 1.5 v/v) and detection was achieved by spraying with phenol-sulphuric acid (Adachi, 1965). The samples were shacked with cation exchange resin, Amberlite IR-120 (H) before applying on silica gel plates (Merck, Darmstadt, Germany). Authentic samples of lactulose; lactose and raffinose were used as reference substances. The unsprayed zoon of the plates, comparing to the authentic samples, were scraped and extracted with 50% methanol then centrifuged the mixtures. The separated pure lactulose and the mixture of oligosaccharides were used for the following experiments.

Colorimetric determination of lactulose

Lactulose was determined colorimetrically by the modification of cystein-carbazole-sulphuric acid methods (Susumu, 1965), in a brief, 0.2 mL of 1.5% cysteine hydrochloride, 6 mL of 70% sulphuric acid, and 0.2 mL of 1.2% carbazole solution in 98% ethanol were added successively to the elute containing 5 to 100 µg/mL of lactulose and mixed in a vortex. The reaction mixture was

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incubated at 37 °C in a water bath for 60 min. the producing color was measured at 560 nm.

Sulfation of oligosaccharides

This was achieved adapting to the method of Hussein (1994) by using chlorosulfonic acid as sulfating agent. The resulted sulfated products were isolated from the reaction mixtures by precipitation with methanol (3 volumes) and purified further by dissolving in water and re-precipitated with methanol.

Biological activities of oligosaccharides

Anti-coagulation activity

The anti-coagulation activity of pure lactulose and the mixture of other oligosaccharides and their sulfated analogs were investigated by using method of USA pharmacopoeia (1960) for the assay of sodium heparin.

Fibrinolytic activities

This was performed by exposing a plasma clot (prepared according to USA pharmacopoeia 1960) to the effect of the investigated samples (at suitable concentration). The lysis percentage of the plasma clots at 37 °C were recorded with each sample and compared with that of standard hemoclar.

RESULT AND DISCUSSION

Effect of time

The results indicated that the reaction time had a significant effect in the biosynthesis of lactulose (Figure 1). The highest yield of lactulose was reached after 7 h (6.89 g/L). Kim *et al*., (2006) reported that the maximum lactulose biosynthesis was achieved after 6 h, while lee *et al*., (2004) found that the maximum (20 g/L) was obtained after 9h. The other oligosaccharides increase to17.2 g/L and 19.68 g/L after 7 h and 10 h respectively.

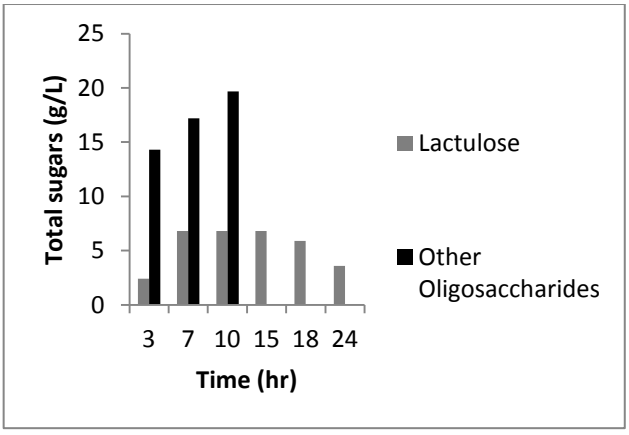

Figure 1: Effect of time course on lactulose and other oligosaccharides productions. The reaction was carried out at the time indicated with 40% lactose and 20% fructose with enzyme concentration 2 U at pH 7.0 and 37 °C.

Effect of enzyme concentration

The results in Figure 2 indicted that, by increasing the enzyme concentration from 2 to 8 U (/mL), lactulose biosynthesis increased to 25 g/L (3.6 times of control) and 15.4 g/L (2 times of the control) after 7 h and 10 h respectively. Increasing the enzyme concentration up to 10 U led to decrease the produced lactulose to 18.48 and 15 g/L after 7 and 10 h respectively. In the contrary of lactulose the other oligosaccharides decreased from17.2 to 12 g/L and from 19.68 to 12.66 g/L by increasing enzyme concentration from 2 U to 10 U after 7 and 10 h respectively (Figure 3).

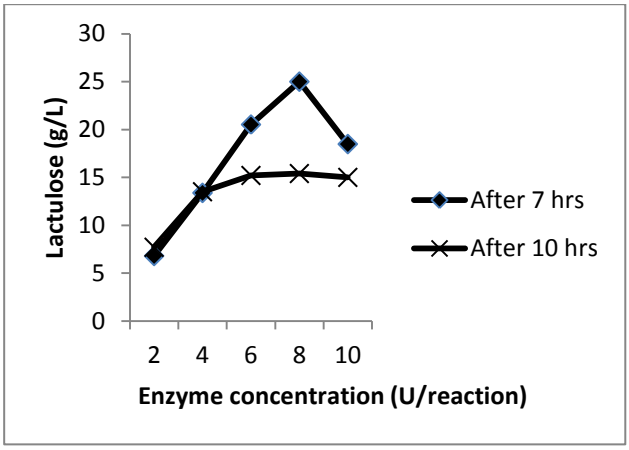

Figure 2: Effect of enzyme concentration on the production of lactulose. The reaction was carried out at different enzyme concentrations with 40% lactose and 20% fructose at pH7.0 and 37 °C.

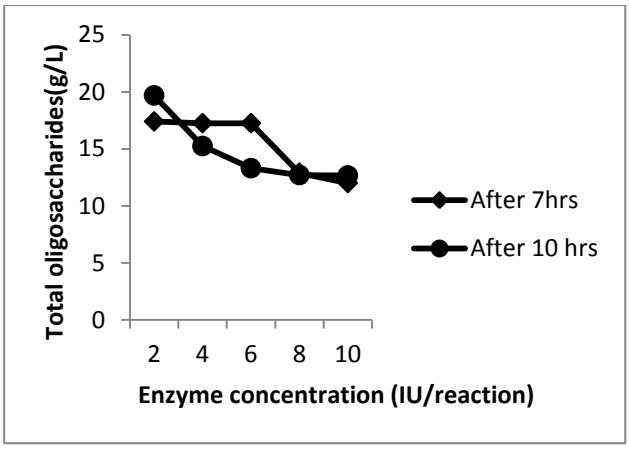

Figure 3: Effect of enzyme concentration on oligosaccharides biosynthesis. The reaction was carried out at different enzyme concentrations with 40% lactose and 20% fructose at pH7 and 37 °C for 7 and 10 hrs.

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These results are in agreement with that reported by Martinez-Villaluenga *et al*., (2008), where they mentioned that trisaccharide amount dominated with the lowest enzyme concentration tested (3 U/mL) while higher enzyme concentration (6 and 9 U/ml) led to rises in disaccharide amount (15% at 9 U/mL) which dominate the GOS mixture.

Effect of pH

The data illustrated in Figure 4 showed that, in reaction mixture contain 40% lactose and 20% fructose and enzyme concentration at 8 U/mL (for 7 h), the optimal pH for lactulose production was found to be 7.0 (25 g/L). The lactulose concentration was decreased in acidic (pH 5.80) and alkali (pH 8.0) condition to 17.8 g/L and 15.4 g/L respectively. It was noticed that by increasing the pH up to 8 the other oligosaccharides increased from 11.45 g/L to 16.8 g/L. These results are in agreement with those reported by Lee *et al*., (2004); Kim *et al*., (2006) and Martinez-Villaluenga *et al*., (2008).

Our results indicated that by using 2, 4 and 6 U at pH7.0, the resulting oligosaccharides were mainly tetra and by increasing the enzyme concentration up to 8 and 10 U, oligosaccharides were penta while at pH 5.5 with 8 U the formed oligosaccharides were trisaccharides.

Effect of substrates concentration

It is well known that one of the main affecting factors in transgalactosylation reaction is the initial substrates concentration (Boon *et al*., 2000; Carlos *et al*.,2011; 2012; Cho *et al*., 2003; Jorgebsen *et al*., 2001). The effect of substrates concentration in this work was indicated in Table 1.

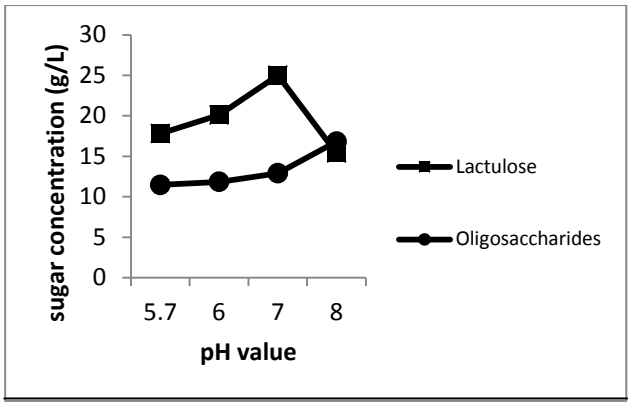

Figure 4: The effect of different pHs on lactulose and oligosaccharides biosynthesis.The reaction was carried out with 40% lactose, 20% fructose and enzyme concentration 8 U at the pHs indicated and 37 °C for 7 hrs.

The results indicated that the maximum concentration lactulose (25 g/L) was achieved by using reaction mixture contain 40% lactose and 20% fructose. Lee *et al*., (2004) and Kim *et al*., (2006) also reported the same substrates concentration as the best one for lactulose production. The lowest yield of lactulose (5.05 g/L) was obtained from the reaction mixture contain 20% galactose; 20% lactose and 20% fructose, but under this condition the high yield from the other oligosaccharides were produced, (17.25 g/L).

Table 1: Effect of substrate concentrations on lactulose and oligosaccharides production.

Substrate Concentrations				Suger Concentrations	
Exp. No.	**Lactose (w/v %)**	**Fructose (w/v %)**	**Galactose (w/v %)**	**Lactulose (g/L)**	**Oligosaccharide (g/L)**
1	20	40	---	16.8	11.3
2	40	20	---	25.0	12.9
3	45	15	---	13.0	12.95
4	50	10	---	16.7	10.7
5	30	30	---	10.3	9.4
6	20	20	20	5.05	17.25

Reactions were carried out at pH 7.0 and at temperature 40 °C with enzyme concentration 8 U/mL for 7 h.

Biological activities

It is well known that lactulose and some others galactooligosaccharides have many applications in the fields of food and pharmaceutical. In this work we found a new application for lactulose and the other produced oligosaccharides and their sulfated derivatives as compounds of high fibrinolytic activities.

The results in Table 2 indicated the highest fibrinolytic activity of the produced oligosaccharides and their sulfated derivatives comparing to that of the standard hemoclar. Unfortunately the produced oligosaccharides and their sulfated derivatives failed to have anticoagulation activities.

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Table 2: Fibrinolytic activities of the isolated lactulose and oligosaccharides samples and their corresponding sulfated products.

Isolated samples	Fibrinolytic activity	
	Before sulfation	After sulfation
Whole samples	**5(+)**	**2(+)**
Lactulose	**6(+)**	**5(+)**
Oligosaccharide	**5(+)**	**5(+)**
Standard*	**3(+)**	

Standard*(hemoclar) 2 mg/mL
3(+):Lysis of 40% plasma clot
6(+): Lysis of 80% of plasma clot.
5(+): Lysis of 70% plasma clot.
4(+): Lysis of 50% of plasma clot.
2(+): Lysis of 25% of plasma clot.

Thus the novel fibrinolytic activity of the produced lactulose and other oligosaccharides and their sulfated derivatives open the possibility of developing a new group of compounds with potential new applications and deserve further clinical assessment and further research.

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