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A BROAD-SPECIFICITY B-GLUCOSIDASE FROM A WILD TYPE OF YEAST ISOLATE AND ITS POTENTIAL USE IN FOOD INDUSTRY

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ABSTRACT

A novel intracellular β-glucosidase was isolated from a wild type of yeast *Trichosporon* sp. Production of enzyme was stimulated with highest level of production in medium containing 1 % lactose as a sole carbon source at pH 7 and 30 ^{o}C for 48 hr. The enzyme preparation exhibits activity towards several β-glucosidic substrates, indicating that, it has broad- specificity; furthermore the activity towards lactose hydrolysis was the highest compared with other substrates. Also, it was able to catalyze transglycosylation. Therefore it was used for milk lactose hydrolysis as well as oligosaccharides production. It has, thus potential use in food industry.

Keywords: *β-Glucosidase, lactose hydrolysis, transglycosylation.*

INTRODUCTION

β-Glucosidase enzymes catalyze the hydrolysis of various compounds with, β -D-glucosidic linkages (Woodward & Wiseman, 1982 and Saha & Bothast, 1996) as well as catalyzing the synthesis of oligosaccharides by reversal of their hydrolytic action (Hansson & Adlercreutz, 2001; Saloheimo *et al*., 2002 & Ishii-Karakasa, 2003). Accordingly these enzymes have been widely utilized in the food industries as a tool for the hydrolysis of glucosidic linkages in various food materials, such as increase in starch recovery from potatoes, extraction of essential oils and the extraction of green tea components. In the flavor industry, β-glucosidases are key enzymes in the enzymatic release of aromatic compounds from glucosidic precursors present in fruits and fermenting products (Shoseyov *et al*, 1990& Gueguen *et al*, 1996). β-Glucosidases, generally categorized as an enzyme which hydrolyses β-(1-4) glucosidic linkage, has a wide variety of enzymatic properties, depending upon the origin and conditions under which the organism was grown(Han & Srinivasan, 1969) β-Glucosidases are divided into three groups on the basis of substrate specificity (I) aryl- β-Glucosidases, which have a strong affinity for aryl- β-Glucosides (II) cellobiases, which hydrolyze only oligosaccharides (III) broad-specificity β-Glucosidases ,which exhibit activity on many substrate types and are the most commonly observed β-Glucosidases (Rojas *et al*, 1995). The β-Glucosidase from the yeast *Trichosporon* sp is broad specificity type since it can hydrolyze many types of β-diglycosides while lactose is the second substrate after cellobiose, thus it can be called enzyme with β-glucosidase and β-galactosidase activity as described previously by (Nakkharat &Haltrich, 2006). They isolated and characterized an enzyme from fungus *Talaromyces*

thermophilus CBS 236.58 followed this phenomenon.Many β-glucosidases with very broad specificity have been isolated from many fungi (Copa-patino & Broda ,1994; Gueguen *et al*, 1995;Kwon *et al* 1992;Pitson *et al*, 1997) According to the classification of β-glucosidases based on substrate specificity (Bhatia *et al*, 2000) most family 1 enzymes also show significant β-glalactosidase activity. β-Glalactosidases catalyse the hydrolysis of lactose; in addition, they were shown to catalyse transgalactosylation reaction. Both the hydrolaze and transferase activity of β-glalactosidases have recently attracted interest because of possible applications in food industry, such as production of low lactose milk and synthesis of oligosaccharides (Cruz *et al*,1999; Karasov *et al*, 2002 , Vasiljvic & Jelen, 2003 , Nakkarat & haltrich,2006) Hydrolysis of lactose by β-glalactosidases alleviates lactose maldigestion problems such as abdominal pain; flatulence or diarrhea which may result from the fermentation of undigested lactose by colonic bacteria with production of H_2, CH_4, CO_2 and short-chain organic acids (Rings *et al*, 1994 ; Fooks *et al*,1999 & Matioli *et al* 2001) Also the hydrolysis of lactose is industrial beneficial to overcome lactose crystallization in condensed milk and ice cream (Karasov *et al* 2002). Oligosaccharides synthesized by enzymes are mainly used as food additives (such as to beverage, infants milk powder, yogurts, chewing gums, and in the manufacture of candy, pastry, bread and jams because of their heat stability), for modification of the coloni microflora toward a healthy balance by increasing the gut bifidobacteria and lactobacilli at the expense of clostridia, proteolytic and *Escherichia coli* bacteroids. This change in the intestinal flora composition has been proposed to be responsible for the decrease of putrefactive products in the feces, for a lower blood cholesterol content, higher Ca 2+ absorption, a smaller loss of bone tissue in ovariectomized rats and a lower incidence of colon cancer (Cruz *et al*,1999; Fooks *et al*, 1999, Sako *et al*, 1999, Boon *et al* 2000, Van Laere *et al*, 2000 , Albayrak & Yang, 2002 & Choi *et al*, 2003) This work presents for the first time a novel enzyme with β-glucosidase and β-glalactosidase activity produced by a newly isolated yeast strain and demonstrates its potential use in the production of low lactose milk and synthesis of oligosaccharides.

MATERIALS AND METHODS

The yeast strain and culture conditions

The yeast strain (*Trichosporon* sp.) used in this study was isolated and identified at Institute of Microbiology and Wine research, Gutenberg University, Mainz, Germany. The medium used for optimization of β-glucosidase production was as follows: 20g Bacto yeast nitrogen base (Difco) with 1% (w/v) carbon source dissolved in 1L of 50 mM buffer solution under studying pH value, media solution were filter sterilized. Erlenmeyer flasks (200-ml) containing 50 ml of medium was inoculated and incubated at test temperature on a rotary shaker (200 rpm) for 24 hr.

β-Glucosidase production

The strain was grown in 5-liter Erlenmeyer flasks each containing 2000 ml of the medium which was optimized (20g Bacto yeast nitrogen base; 1% (w/v) lactose at pH 7 and 30°C for 48 hr). Cells were harvested by centrifugation

2000Xg, 15 min, washed twice with 50 mM sodium citrate buffer pH 5 and centrifuged again. The resultant paste was stored at – 18 °C until used.

Preparation of β-Glucosidase

The cell paste was suspended in appropriate volume of 50 mM sodium citrate buffer pH 5 and homogenized with one volume of glass beads (diameter 0.5 mm). Cells were disrupted by shaking in vibrating homogenizer (Vibrogen Zellmühle, E. Bühler, Tübingen, Germany.) at 4°C for 6 min (Mireau *et al*, 2000). Debris was removed by centrifugation at 20000X g for 30 min, supernatant was desalted by dialysis against distilled water and lyophilized. For partial purification the lyophilized preparation was dissolved in 20 mM Tris/HCl buffer, pH 7.6 and loaded onto a column (containing Q Sepharose Fast Flow, Pharmacia) which previously was equilibrated with 20 mM Tris/HCl buffer, pH 7.6 Elution was carried out with a linear gradient of 2M NaCl in the same buffer, at a flow rate of 1 ml/min. Active fractions were combined, desalted, lyophilized and used as enzyme preparation for further study.

Enzyme assay

One ml of 5 % lactose (0.139M) solution or other tested saccharides in 0.05M phosphate buffer pH 6.5 was mixed with the enzyme solution in total volume of 2.0 ml and incubated at 40°C. The reaction was stopped after 1 hr by heating the tubes in a boiling water bath for 5 min, One ml of the reaction mixture was deproteinized by adding 1.0 ml of 5 % $ZnSO_4$ $7H_2O$ and 1ml of 4.5% $Ba(OH)_2$ and filtered. Glucose produced was estimated in 1ml of the filtrate by the glucose-oxidase method as cited by Cruz *et al*, 1981. With nitrophenylglucoside as substrate, the *p*-nitrophenol release at 40 °C was monitored at 400 nm. In both cases an enzyme unit (U) was defined as the amount of enzyme necessary to liberate 1µmol of the glucose or *p*-nitrophenol per min under the assay conditions (Makkar *et al*, 1981)

Thermal stability

The enzyme solution (3 units) was incubated in 50 mM citrate buffer pH 5 at different temperatures ranging from 4 ^oC to 80 ^oC for 60 min., and then the remaining activities were determined using lactose as the substrate (Oikawa *et al*, 1998)

pH stability

The enzyme solution (3 units) was treated with various buffers at 4 °C for 3 h., then the pH was readjusted to 6.5, then the remaining activities were determined using lactose as the substrate. The following buffers (50 mM) were used: (a) glycin / HCl, pH 2 – 3, (b) citrate pH 4 - 5, (c) sodium phosphate pH 6 - 7, (b) Tris / HCl pH 8 and glycin / NaOH pH 9 – 10 (Oikawa *et al*, 1998).

Milk sample

Milk sample was obtained from the farm of the Faculty of Agriculture,Kafr Elsheikh. It has the following composition, 3.3% fat, 4.7% lactose, and 0.71 % ash. For whole milk the sample was used without treatment. Skim milk sample was prepared by centrifuging the cold whole milk at 8000Xg for 30 min then the fat was removed.

Oligosaccharide synthesis

The basic reaction mixture containing enzyme preparation (2 units) and 1ml of 250 mM lactose at tested pH values and temperatures was left for 16 h, and then boiled for 5 min in water bath, and centrifuged at 1000X g for 3 min. The supernatants were filtered through syringe filter (0.45 µm Minisart, Sartorius) and analyzed by HPLC equipped with an Aminex column HPX-42A; (300 mm x 7.8 mm). Twenty µl of each sample was applied onto the column and eluted with deionized water at a flow rate of 0.6 ml/min. The reaction products were detected by refractive index and identified and quantitated by comparison with retention times of authentic appropriate sugars standards (Modified method of Choi *et al*, 2003)**.**

RESULTS AND DISCUSSION

Optimization of β-glucosidase production

Since β-glucosidase properties are dependent upon the origin and conditions under which the organism was grown (Han &Srinivasan,1969) while the nutritional and environmental factors for β-glucosidase production by the used strain was not previously known, thus the purpose of these growth studies was to determine the conditions that stimulate the enzyme production as follows:

Effect of growth temperature on enzyme production.

The strain was cultivated at a temperature range of 10–50 °C, for 24 hr in medium containing 1% (w/v) cellobiose as a carbon source at pH 7 to determine the optimal temperature for enzyme production, the results were summarized in Fig 1. The yeast was able to grow and release β-glucosidase at a wide range of temperature. The maximum enzyme yield (5.7U/ml) was produced at 30 °C.

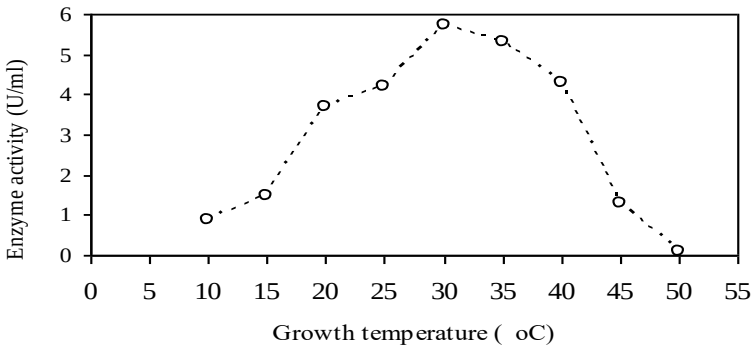

Fig 1 Effect of cultivation temperature on enzyme production.

Effect of initial pH of culture medium on enzyme production.

The strain was cultured at various pH values from 3 to 10, for 24 hr in medium containing cellobiose as a carbon source at 30 °C. Fig 2 shows that

the yeast was able to grow and produce the enzyme at a broad range of pH, while the highest enzyme yield (5.9 U/ml) was formed at pH 7.

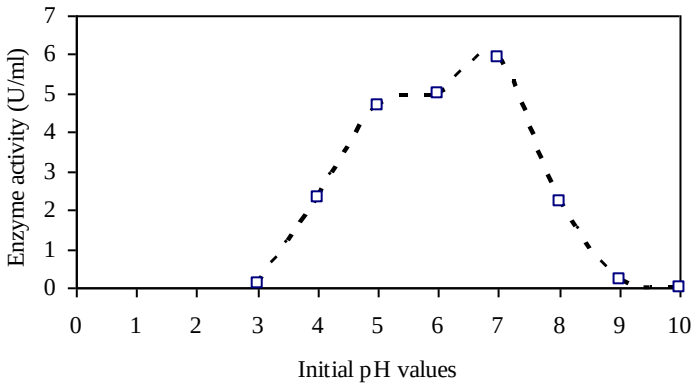

Fig 2 Effect of medium pH on enzyme production

Effect of carbon source on enzyme production

Various β-glucosidic and non-β-glucosidic carbohydrates were employed in equal concentrations of 1% (w/v) to test their ability for inducing the formation of β-glucosidase (Table 1). In general, the yeast can utilize all tested carbohydrates with differences in the enzyme production. The formation of highest amount of β-glucosidase was induced by lactose. It could be also observed that the enzyme formation is not dependent on the kind of glucosidic bond since cellobiose, maltose and isomaltose induced the formation of approximately the same enzyme yield. These results are in agreement with the observation of other workers (Skory *et al*, 1996 and Riou *et al*, 1998) for optimization of β-glucosidaes production from other organisms.

Table 1: Effect of various carbohydrates on enzyme production.

Carbon source	β-glucosidase activity (U/ml)
Glucose	5.5
Salicin	5.3
Maltose	26.6
Isomaltose	26.1
Raffinose	2.1
Lactose	31.0
Cellobiose	26.8
Sucrose	6.1

Time course of β-glucosidase production

The yeast strain was cultivated under the above-identified optimal cultural conditions for 72hr. Fig. 3 shows that the enzyme activity (0.9U/ml) was detected after 6hr of incubation and increased gradually to reach its maximum (33.45U/ml) within 48 hr of the cultivation, and then started to decrease.

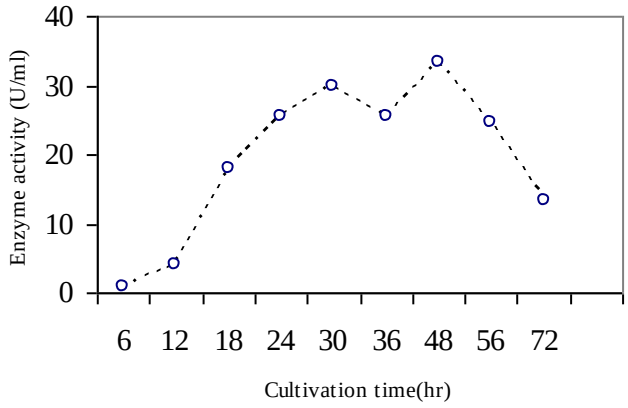

Fig 3 Effect of incubation periods on enzyme production

Thermal stability

The thermal stability experiments results are shown by Fig 4, which reveals that the enzyme preparation maintains its activity up to 35°C and even at 40 °C, it retains 86.1 % of its maximum activity at 45 °C and dropped rapidly, to be completely inactivated at 80 °C.

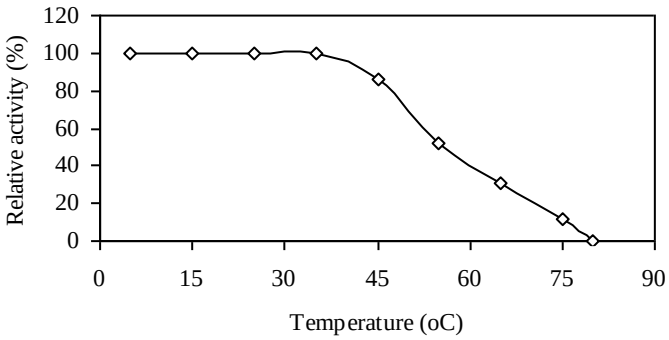

Fig 4 Effects of temperature on the enzyme stability.

pH stability

The enzyme preparation was quite stable at pH 5 to 6 Fig 5. The activity was gradually retarded above and below the mentioned values.

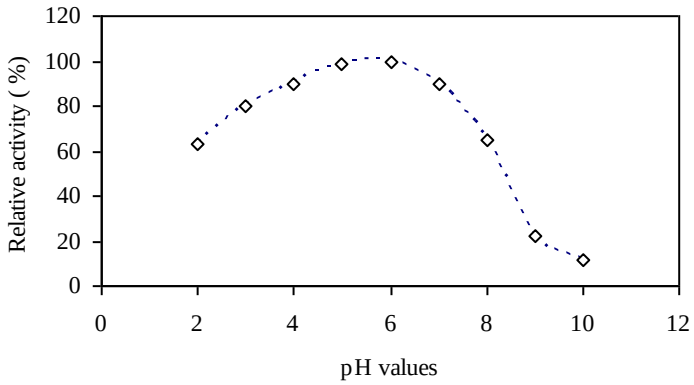

Fig 5 Effects of pH on the enzyme preparation stability.

Substrate specificity of β-glucosidase preparation

The enzyme preparation exhibited a broad specificity for hydrolyzing various carbohydrate types (Table 2). The rate of hydrolysis depended on the nature of aglycon moiety and the type of linkage. Highest hydrolyzing activity was observed with substrate having β-(1,4)-glucosidic linkages, such as cellobiose and lactose. The enzyme could also hydrolyze the saccharides with β- (1,6) glucosidic linkages effectively. The enzyme, however, had very little (28.7; 3.2% and 0%) or no activity on sophorose, raffinose, and sucrose respectively. On the other hand, activity against polysaccharides carboxymethylcellulose (CMC) and acid-swollen avicel was not found. From the above results it can be suggested that the enzyme belonged to class " Broad substrate specificity β-glucosidase enzyme " according to the classification system based on substrate specificity (Bhatia *et al*, 2002). β-Glucosidases with very broad specificity have been isolated from many bacteria, yeast and fungi (Han and Srinivasan,1969;Wood and McCrae,1982 and Yan and Lin, 1997). The enzyme efficiency towards lactose hydrolysis paid our attention to do additional studies about its catalytic ability.

Table 2: Relative values rates of hydrolysis of various substrates by the enzyme preparation.

Substrate	Type of linkage	Relative hydrolysis rate (%)
p-Nitrophenyl-β-D-glucopyranoside	βGlc	100
Sucrose	β,α (1,2)	00.0
Lactose	β(1,4)Gal	119.3
Salicin	βGlc	18.0
Raffinose	α(1,6), β (1,2)	3.2
Sophorose	β (1,2)Glc	28.7
Gentiobiose	β (1,6)Glc	79.0
Cellobiose	β(1,4)Glc	131
carboxymethylcellulose	Polyβ(1,4)Glc	00.0
Avicel	Polyβ(1,4)Glc	00.0

Milk lactose hydrolysis.

The milk is consumed in two preferred forms skim and whole milk containing normally about 4.7 % fat, thus this study aimed to define the optimum conditions for hydrolysis in both forms. The main factors, which affect on enzyme activity, include reaction pH, reaction temperature, substrate concentration, etc. Since the substrate concentration (lactose in raw milk) is approximately stable, the study was focused on the other mentioned factors.

Effect of temperature on milk lactose hydrolysis by enzyme preparation

To define the effect of temperature on enzyme activity, the enzyme was incubated with milk at temperatures ranged from 30 to 50 ^{o}C. The results (Table 3) reveal that, the reaction was normally affected by temperature and the maximum hydrolysis yield was found at 35 ^{o}C with both milk forms above, then a gradual fall in the hydrolysis yield was occurred. In comparison with other yeasts (*Kluyveromyces fragilis* and *Kluyveromyces lactis*) enzymes, used for lactose hydrolysis, they showed optimum temperature at 40 ^{o}C (Matioli *et al*, 2001 and Genari *et al*, 2003).

Table 3 Effect of temperatures on milk lactose hydrolysis.

Temperature ^{o}C	Lactose conversion % in whole milk	Lactose conversion % in skim milk
30	35.90	36.85
35	38.23	40.32
40	30.00	32.13
45	26.33	28.66
50	21.61	23.51

Effect of reaction pH on milk lactose hydrolysis.

The results (Table 4) indicated that the enzyme was capable to hydrolyze milk lactose at various tested pH values, while luckily the maximum activity was coincided with the pH value (6.5) of the natural milk.

Table 4: Effect of reaction pH on milk lactose hydrolysis.

pH values	Lactose conversion % in whole milk	Lactose conversion % in skim milk
5.5	27.00	27.35
6.0	27.80	29.11
6.5	29.19	31.91
7.0	26.04	27.43
7.5	21.11	24.22

This property allows using the enzyme in dairy industry without modification of milk pH. These results are in agreement with that of Makkar *et al*, (1981) & Matioli *et al*, (2001), they reported that pH 6.5 was the optimum value for lactose hydrolysis by the enzymes from *Kluyveromyces fragilis* and *Lactobacillus bulgaricus*.

Time course of milk lactose hydrolysis.

Various enzyme concentrations from (2 U/ml to 5 U/ml) were used to hydrolyze the lactose in both skim and whole milk at above estimated optimum temperature and pH for different time periods from 1hr to 5hr to determine the minimum enzyme concentration which reduce the lactose content to be satisfactory for lactose intolerance at minimum time to prevent any change in milk properties during the treatment. The experimental results (Table 5) demonstrated that, in general the lactose hydrolysis increased by increasing of both the enzyme concentration and reaction time. The activity towards hydrolysis of skim milk was slightly more than that of whole milk at all determinations. Since it is satisfactory for lactose intolerants to hydrolyze of 90 % of lactose in a products contains about 50 g /l lactose (Hernandez & Asenjo, 1982). Therefore based on the experimental results (Table 5) the whole and skim milk treated with amount of enzyme 5U/ml for 4 hr was satisfactory for lactose intolerants. On the other hand, skim and whole milk treated with 4 U/ml for 3hr was readily accepted in point of view of industrial conditions according to the recommendation of Prenosil *et al*, 1987. They indicated that hydrolysis of 75 % - 85% of lactose in milk containing 5 % lactose is industrially accepted. In comparison, with other enzymes from other organisms, the use of 3450 U/l β-galactosidase from *Kluyveromyces fragilis* due to a conversion of 70 % of lactose from substrate contain 5% (w/v) lactose at pH 6.5 and 40 ^{o}C in 2 hours (Matioli *et al*, 2003).

Table 5 Time course of milk lactose hydrolysis.

Time (hr)	Whole milk				Skim milk			
	Enzyme concentrations				Enzyme concentrations			
	2U/ml	3U/ml	4U/ml	5U/ml	2U/ml	3U/ml	4U/ml	5U/ml
1	18.00	23.33	28.10	30.12	19.19	22.91	29.08	31.90
2	37.44	46.16	56.54	65.67	38.00	47.51	57.90	66.60
3	45.16	57.74	71.80	84.90	45.80	58.42	71.90	86.12
4	52.88	69.32	87.42	92.27	53.00	70.31	88.61	93.05
5	60.61	70.90	90.15	96.10	60.90	78.00	90.11	95.23

Oligosaccharides formation

Effect of temperature on the synthesis of oligosaccharides

To determine the optimum temperature of oligosaccharides formation, the reaction was carried out at temperature range 20 - 60 ^{o}C. The results (Table 6) showed that the formation of oligosaccharides increased with the increase of temperature to reach its maximum at 40^{o}C and then started to decline. These results are in agreement with the observation of Monsan & Paul, 1995; Vasiljevic & Jelen, 2003) they reported that the amount and the rate of oligosaccharides formation were significantly affected by the reaction temperature and pH.

Effect of pH on oligosaccharides formation.

To define the optimum pH for oligosaccharides synthesis, the reaction pH was adjusted in the pH range 4 – 9. The results (Table 7) showed that, the highest amount of oligosaccharides was formed at pH 8. It could be

also noted that, the optimum temperature and pH for lactose hydrolysis by the same enzyme were 35 ^{o}C and 6.5(Tables 3 and 4) while in case of oligosaccharides synthesis they were shifted to be 40 ^{o}C and pH 8, this observation was previously mentioned by Cruz *et al*, 1999.

Table (6): Effect of temperature on galactooligosaccharide synthesis.

Temperature °C	Reaction products (mM)			
	Glucose	galactose	Lactose	Galactooligosaccharide
20	95.25	84.01	158.84	0.71
30	152.53	107.05	118.68	1.21
40	128.75	99.42	134.59	3.88
50	125.12	95.28	140.64	1.01
60	47.14	43.01	204.81	0.0

Reaction conditions: substrate concentration 250mM; Enzyme, 2U/ml and pH 7 for 16hr.

Table (7): Effect of reaction pH on galactooligosaccharide synthesis.

Reaction pH	**Reaction products (mM)**			
	glucose	**galactose**	**lactose**	**galactooligosaccharide**
4	70.28	68.01	179.85	0.04
5	116.00	99.05	138.89	1.80
6	124.76	120.92	122.66	3.00
7	125.11	118.28	121.59	4.01
8	121.04	110.91	125.81	4.68
9	49.99	46.85	199.82	1.62

Reaction conditions: substrate concentration 250mM; Enzyme, 2U/ml and Temperature, 40 ^{o}C for 16hr. The following buffers (50 mM) were used: (a) citrate pH 4 - 5, (b) sodium phosphate pH 6 - 7, (c) Tris / HCl pH 8 and glycin / NaOH pH 9.

Effect of substrate concentration on oligosaccharides formation.

In order to test the influence of lactose concentration on the synthesis of the galactooligosaccharides, enzyme at concentration of (2U/ml) incubated with various lactose concentrations. The results (Table 8) reveal that, in general this novel enzyme was able to catalyze transgalactosylation reaction with lactose as a substrate. The formation of oligosaccharides was absolutely dependent on lactose concentration, at lactose concentration lower than 200 mM, the hydrolysis reaction was dominated, but above the mentioned concentration, the reaction was shifted towards the formation of oligosaccharides. This may be due to that, β-galactosyl groups have a higher ability of attaching to lactose than water as an acceptor at increasing lactose concentration (Iwasaki *et al* 1996). The formation of oligosaccharides was gradually increased with the increasing of lactose concentration to reach its maximum at 400 mM. These observations are in accordance with the findings of Boon *et al*, 2000, Vasilijevic & Jelen, 2003; they reported that, initial lactose concentration is the most significant factors affecting on the oligosaccharides synthesis. Data also reveal that, the yield of oligosaccharides obtained here was lower than that obtained by other workers from other microbial enzymes (Rabiu *et al*, 2001, Albayrak &yang,

2002, & Choi *et al*,2003). This may be due to that, oligosaccharides synthesis reaction activated by our enzyme preparation still need more optimization by studying the other parameters affecting the oligosaccharides formations such as enzyme concentration, reaction time, activators, inhibitors, etc. However, our results cleared for the first time, this novel enzyme is capable of producing oligosaccharides by transgalactosylation, which have high biological value. Also our results indicated that, the produced yield of oligosaccharides was able to enhance by optimizing the factors, which affect the reaction such as temperature, pH and substrate concentration.

Table (8) Effect of substrate concentration on galactooligosaccharide synthesis

Substrate concentration (mM)	Reaction products (mM)			
	glucose	galactose	lactose	galactooligosaccharide
100	99.87	98.92	0.0	0.0
200	166.54	131.46	48.58	2.44
300	224.29	158.8	98.89	6.84
400	264.95	179.54	158.61	13.82

Reaction conditions: pH 8; Enzyme, 2U/ml and Temperature, 40 oC for 16hr.

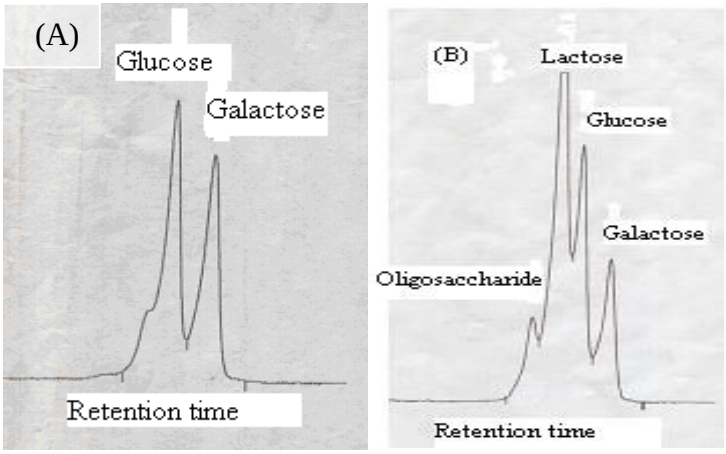

Fig (6): A typical HPLC Chromatogram showing (A) lactose hydrolysis at low lactose concentration (B) oligosaccharide formation at high lactose concentration

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إنتاج إنزيم البيتاجلكوسيديز واسع النشاط من سلالة خميرة برية و إمكانية استخدامه في مجال الصناعات الغذائية

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أمكن عزل إنزيم البيتا جلوكوسيديز لأول مرة من خميرة ال *Trichosporon* sp عندما تنمو في ظروف مثالية في بيئة درجة حموضتها ٧ و تحتوي علي ١ % لاكتوز كمصدر وحيد للكربون لمدة ٤٨ ساعة علي درجة حرارة ٣٠ درجة مئوية. و وجد أن مستحضر الإنزيم له نشاط تجاه العديد من المركبات الجليكوسيدية ذات الرابطة بيتا وكان أعلي نشاط له تجاه سكر اللاكتوز لذا فإنه استخدم لخفض نسبه سكر اللاكتوز في اللبن كما استخدم لتخليق سكريات الأوليجو ذو القيمة الحيوية العالية من اللاكتوز لذا فهو ذو قيمة تطبيقية عالية في مجال الصناعات الغذائية.