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ORIGINAL ARTICLES

Isolation, Screening, Identification and Optimization of Cultural Conditions for Selected Local Bacterial β-Galactosidase Producer

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ABSTRACT

Fifty two bacterial isolates were isolated from different sources (commercial cow milk "from markets present in Giza", commercial yoghurt "from markets present in Giza" and agricultural soil" presents in Giza and Kalubeia") and assessed for their β-galactosidase production. Results of this work showed that 24 of these isolates produced β-galactosidase. The most producer bacterial isolate was identified as *Bacillus subtilis*. In addition, some of nutritional and environmental conditions for production of the enzyme by the selected isolate were studied of modified media contain as. Lactose 5g/L, yeast extract 10g/L, pH of the medium 7.0 and the incubation temperature at 30°C for 48 hours was the most suitable conditions for the production of β-galactosidase.

Key words: β-galactosidase*, Bacillus* sp., Lactase deficiency.

Introduction

Lactose maldigestion is the most common disorder of intestinal carbohydrate digestion in humans, and is a widespread problem occurring in approximately 70% of the world's population(Savaiano & Levitt, 1987).

Lactase deficiency is due to the reduction or loss of lactase activity in the intestinal brush border. The passage of lactose to the large intestine can lead to tissue dehydration, poor calcium absorption, generation of hydrogen and carbon dioxide gases, abdominal pain, diarrhea, bloating, flatulence, blanching, and cramps (Chen *et al.*, 2009).

β-Galactosidases (lactase) hydrolyze lactose, the sugar in milk, into its components, glucose and galactose, allowing milk and its derivatives to be treated for consumption by people with lactose intolerance. β-Galactosidases also have other applications, such as the prevention of lactose crystallization in frozen and condensed milk products, reduction of water pollution caused by whey, and increasing the sweetening properties of lactose (Soliman, 2008).

On the other hand, this enzyme can also catalyze a transgalactosylation reaction, which leads to the formation of di-, tri-, or higher galacto-oligosaccharides (GOS). GOS were found to stimulate the growth and establishment of bifidobacteria in the human intestine and suppress potentially harmful bacteria such as clostridia and Bacteriodes species in the gut and as such are now regarded as a prebiotic food ingredient (Hsu *et al.*, 2007).

β-Galactosidase is widely distributed in Nature and is produced by animals, plants and micro-organisms(Todorova-Balvay, *et al*., 2006; Somyos & Phimchanok, 2009).

Enzymes of plants and animal origin have little commercial value but several microbial sources of *β*-galactosidase are of great technological interest. Microorganisms offer various advantages over other available sources such as easy handling, higher multiplication rate, and high production yield. As a result of commercial interest in *β*-galactosidase, a large number of microorganisms have been assessed as potential sources of this enzyme (Panesar *et al.*, 2010). Although there are many commercial β-galactosidases produced, mainly from yeast and fungi, the practical application of these enzymes is still faced with many technical problems. From these, the most of the known β-galactosidases (optimum temperatures above 30°C) do not have good activity for hydrolysing lactose at low temperatures of 0-10°C at which milk is usually kept and stored to prevent spoilage (Asraf & Gunasekaran, 2010).

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In addition, the microorganisms used to produce the β-galactosidases have varying nutritional requirements and thus they produce enzymes other than β-galactosidases such as proteolytic and lipolytic enzymes which can produce inferior organoleptic properties or other quality defects in milk/milk products (Grieve *et al.*, 1983).

Consequently, there is a need for new microbial sources that are capable of producing economic quantities of β-galactosidases with the ability to function efficiently at high or low temperatures.

The present study was designed to isolate, screen different bacterial isolates from local sources for their production to β-galactosidase, identify of the most active bacterial isolate and to determine the most suitable conditions for the production of this enzyme.

Materials and Methods

Bacterial Isolates, Culture Media And Growth Conditions:

Fifty two bacterial isolates isolated from different sources; cow milk(from different markets present in Giza), 22 isolates; commercial yoghurt(from different markets present in Giza), 9 isolates and agricultural soil(from Giza and Kalubeia), 21 isolates) at local area.

All isolates were grown on nutrient agar medium (Oxoid) by incubation at 37˚C for 24 hours and stored at -70˚C. Other culture medium was used as a basal medium to determine the optimal conditions for the production of β-galactosidase which was contained, per liter: Lactose, 5g; Peptone, 5g and Beef extract, 3g(basal medium).

Screening Of Β-Galactosidase Producing Isolates:

The screening of the β-galactosidase producing isolates was carried out by using o-Nitrophenly β-D-galactoprynoside (ONPG) as substrate; this test was done according to (Miller, 1972)**.**

Enzyme Assay:

The extra- and intra-cellular β-galactosidase activity was determined. For extracellular enzyme, cells were removed from the culture broth by centrifugation at 4,000Xg for 20 min at 4°C. The cell-free culture supernatant was stored at 4°C until used. For intracellular enzyme, grown cells were harvested in a cooling centrifuge at 4,000Xg then washed three times with cold 0.1 M phosphate(pH 6.5) buffer, resuspended to a volume of 50 ml, the cell suspensions were sonicated on ice in glass tubes using a Branson Sonic Power Sonicator (48 Bransonic Power,40W, 30 sec with 30 sec cooling periods) for 4min,Then they were centrifuged at 4000xg (in a cooling centrifuge) for 20min (Song and Jacques,1997),and the supernatant was used for measuring β-galactosidase activity Subculture of isolate(s) was carried out on 5 ml of fresh lactose broth and incubated at 37°C overnight. Four drops of fresh subculture of the isolate(s) were added to tubes containing 5 ml of lactose broth and re-incubated at 37°C for 2-3 hours to measure their optical density at 600 mμ (OD_{600}), (which must 10^8 cells/ml).these tubes were cooled to prevent further growth by immersing in an ice bucket containing a mixture of ice and water. For measuring the enzymatic activity of β-galactosidase, 0.5 ml of the culture broth was mixed with 0.5 ml of Z-buffer, then addition of One drop of 1%SDS and two drops of chloroform was done,vortex the tubes for 10 seconds. these tubes were incubated at 37°C for 5 min then 200µl of ONPG(4 mg in ml of 0.05M phosphate buffer pH6.0) was added and shaked for a few seconds. the time of the reaction was recorded with a stop watch, the reaction was Stopped by adding 0.5 ml of 1M Na_2 CO_3 solution after sufficient yellow color was developed. the optical density was recorded at both 420 mμ and 550 mμ, using a standard calibration curve. One unit of β-galactosidase activity (U) was defined as the amount of enzyme that liberates 1 µmole ONP(o-Nitro phenyl) per minute under assay conditions**.**

Assay Of Protein Concentration:

The protein concentration was determined by the Lowry method by using bovine serum albumin used as a standard (Lowry *et al.*, 1951).

Identification Of The Most Producer Strain:

The following morphological and biochemical tests were performed to identify the selected strain: Cell shape, size, shape of end and chain of tested isolate were studied on fresh nutrient agar culture (24- 48 h at 37 ± 2^{o}C), spore shape, position, presence of parasporal bodies and swelling of sporangium by Gram staining technique and light microscope examination, the motility by using *Malachite* green technique. Physiological tests were carried out on the experimental isolate : its ability to grow at different temperature between(5.0 -65 $^{\circ}$C), pH levels (5.0-10.0) and concentrations of NaCl (0.0-10.0%) on nutrient broth medium. The ability of acid production from different carbohydrates (D-glucose, L-arabinose, D-mannitole, D-mannose, salicin, starch & D-xylose) by tested isolate was investigated in broth nutrient medium. Hydrolysis of starch, tween 20 & 80, casein, egg yolk and gelatin were studied in nutrient agar medium (except gelatin in nutrient broth medium) supplemented with 5% of the specific substrate. Nitrate reduction by the tested isolate was studied in nitrate broth medium The reaction of the tested isolates on *Voges–Proskauer test was studied on* methyl red Voges-Proskauer broth medium **(**Atlas, 2004).According to the criteria described in Bergey's Manual of Systematic Bacteriology (Logan & De Vos,2009).

Effect Of Incubation Period:

The effect of incubation period was determined by incubating production medium for different incubation periods (24, 48 and 72 hours) at 37°C. the cells fresh weight,the enzymatic activity,total protein were carried out for both extracellular and intracellular enzyme.

Effect Of Different Incubation Temperatures:

The optimum temperature was determined by incubating at different temperatures the selected isolate (20, 25, 30, 37, 40, and 42°C) for 24 hours. After incubation period with certain temperature, the cells fresh weight, the enzymatic activity and total protein were carried out for both extracellular and intracellular enzyme.

Effect of Different pH Values:

The optimum pH for *production of* β-galactosidase was studied by inoculated the selected isolate in lactose yeast extract broth medium which was prepared at different initial pH values, ranged from 4.0 to 9.0 using 6N HCl and 6N NaOH and after incubation period, the cells fresh weight, the enzymatic activity,total protein were carried out for both extracellular and intracellular enzyme.

Effect Of Carbon Sources:

Different carbon sources such as lactose, sucrose, glucose, fructose, starch, galactose, dextrane, and inositol were employed to find the suitable carbon source for β-galactosidase production by selected strain. All these sources were studied as that of basal medium (10g/L).

Effect Of Nitrogen Sources:

Two categories, organic nitrogen sources and inorganic nitrogen sources were employed. The growth medium was initially supplemented with different organic nitrogen sources, i.e. yeast extract, peptone, casein, casein hydrolysat, glycine and Beef extract. Among the inorganic nitrogen sources, potassium nitrate, sodium nitrite, ammonium sulphate, ammonium nitrate, ammonium chloride and ammonium sulphate. All these sources were studied as that of basal medium (5g/L).

Results and Discussion

Isolation From Different Sources:

52 isolates were obtained from 3-different sources: 22 isolates from commercial cow milk, 9 isolates from commercial yogurt and 21 isolates from agricultural soil; they were cultivated on basal nutritional medium.

Screening Of Bacterial Isolates:

The screening of the bacterial isolates to produce β-galactosidase. As shown in table,1 Out of 52 bacterial isolates, 24 bacterial isolates(soil, 8 isolates ; cow milk,10 isolates and yogurt, 6 isolates) were shown β-galactosidase activity. The bacterial isolate No.12 which obtained from agricultural soil, gave the highest enzymatic activity. This isolate was identified and used for further investigations.

Identification Of Bacterial Isolate:

The most β-galactosidase producer bacterial isolate No.12 was identified according to the criteria described in Bergey's Manual of Systematic Bacteriology (Logan & De Vos,2009)., using phenotypical and biochemical characteristics. The result showed that this isolate was identified as *Bacillus subtilis*. it is aerobic, Gram positive, motile rods, forming spherical & ellipsoid spore which terminal in unswollen sporangium which empties from parasporal crystals. It gives a positive reaction with catalase test, production of ß-*galactosidase*, *Voges–Proskauer* test, utilization of citrate, hydrolyses of gelatin and production of acid from D-glucose, L-arabinose, D-mannitole, D-mannose, salicin & D-xylose. Moreover, it can grow at different pH levels from 5.0 to 8.0, temperature degrees from 20.0 to 40.0 ±2 °C and concentrations of NaCl from 0.0 to 5.0%. In contrary, it gives a negative reaction with hydrolyses of tween 20 & tween 80, production of acid from starch, nitrate reduction, growing at pH levels 9.0 &10.0, NaCl 7.0 & 10.0% and temperature degrees 55.0 & 65.0 ±2 °C.

Table 1: The enzymatic activity of the obtained bacterial isolates quantitatively.

Source of isolates	code of the isolate	The obtained activity(UI/ml)
soil	Isolate No.3	414
	Isolate No 6	317
	Isolate No 7	257
	Isolate No 8	256
	Isolate No 9	130
	Isolate No 12	1379
	Isolate No 13	903
	Isolate No 24	834
Milk	Isolate No 10	369
	Isolate No 11	298
	Isolate No 14	125
	Isolate No 15	297
	Isolate No 16	258
	Isolate No 17	450
	Isolate No 19	286
	Isolate No 20	322
	Isolate No 21	465
	Isolate No 25	215
Yogurt	Isolate No 37	440
	Isolate No 38	386
	Isolate No 44	758
	Isolate No 50	126
	Isolate No 51	213
	Isolate No 52	250

Effect Of Environmental And Nutritional Conditions On The Production Of Β-Galactosidase:

Presented data showed that the isolated bacteria isolate No.12 produce β-galactosidase. The optimal conditions for β-galactosidase production were determined under submerged fermentation conditions.

-Effect Of Incubation Period On The Enzyme Production:

It was determined that the optimum incubation time for maximum production of β-galactosidase (extracellular and intracellular) by *Bacillus subtilis* (isolate No.12) was at 48h (Table,2). A prolonged incubation time beyond this period did not increase the enzyme yield.

Table 2: Effect of incubation period on the production of enzyme by bacterial isolate No.12.

Incubation time (Day)	Extracellular Enzyme			Intracellular Enzyme			Cells fresh weight(mg/ml)
	Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	
1	336	1156	0.290	960	1680	0.571	31
2	440	1366	0.322	1120	1799	0.733	40
3	167	955	0.174	750	1487	0.504	27

The reason for this might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium and probably due to depletion of nutrients available to microorganism (Ramesh & Lonsane, 1987, Akcan *et al.*, 2011).

-Effects Of Different Incubation Temperatures On The Production Of Enzyme By Bacterial Isolate No.12.

Using of wide variety in the incubation temperature was done starting from 20°C to 42°C with using submerged culture.

Results presented in table 3 showed that 30°C was the optimum temperature for β-galactosidase production (extracellular and intracellular) by bacterial isolate No.12 as shown at higher temperature (40-42°C) a clear decreasing in enzymatic activity.

Table 3: Effect of incubation temperature on the production of enzyme by bacterial isolate No.12.

Incubation temperature	Extracellular enzyme			Intracellular enzyme			Cells fresh weight(mg/ml)
	Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	
20	120	1230	0.097	230	387	0.60	18
25	250	1400	0.178	400	677	0.60	21
30	430	1642	0.261	1430	854	1.70	34
37	380	1566	0.242	1290	866	1.50	31
40	130	1220	0.106	200	412	0.50	6
42	50	1110	0.045	80	228	0.35	1

These data were almost agreed with several authors, (Nizamuddin *et al.*, 2008, Murad *et al.*,2011) who reported that the optimal temperature of highest enzymatic activity was 30°C**.**

-Effects Of Different Ph Values On The Production Of Enzyme By Bacterial Isolate No.12.

Controlling the pH of the culture during fermentation has been reported to enhance microorganism growth and enzyme production (Chou *et al.*, 1999).

Results as shown in table 4 showed that the highest β-galactosidase activity was obtained when the initial pH of the medium was adjusted to 7.0 (extracellular and intracellular). While the activity declined sharply on either side of this optimal range.

Table 4: Effect of different initial pH on the production of enzyme by bacterial isolate No.12.

Initial pH	Extracellular enzyme			Intracellular enzyme			Cells fresh weight(mg/ml)
	Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	
4	127	1176	0.107	258	1327	0.194	6
5	166	1233	0.134	480	1509	0.318	13
6	219	1389	0.157	609	1709	0.356	33
7	405	1643	0.246	1390	1809	0.768	54
8	310	1490	0.208	790	1691	0.467	15
9	0	0	0	0	0	0	0

In this connection Ismail *et al.*, (2010) reported that the optimum initial pH was 7.0 to get on the maximum enzymatic activity for *L. acidophilus* NRRL-4495. Gumgumjee & Danial, (2011) stated that the optimal initial pH in the medium for β-galactosidase production was 6.0 while the cells of the isolated bacteria strain grew best at initial pH7.0.The results also showed that when initial pH was higher or lower than 6.0, β-galactosidase activity decreased sharply, indicating that β-galactosidase production by the *Bacillus licheniformis* E66 strain was very sensitive to the change in initial pH. While Hsu *et al.*, (2005) stated that initial pH was optimum at 6.5 which giving highest enzymatic activity and productivity for *Bifidobacterium longum CCRC 15708*

-Effect Of Carbon Sources On The Enzyme Production:

The nature and amount of carbon source in culture media is important for the growth and production of extracellular β-galactosidase in bacteria. Carbon source regulates biosynthesis of β-galactosidase in various microorganisms (Hsu et al., 2005, Konsoula & Liakopoulou-Kyriakides, 2007, Alazzeh *et al.*, 2009). All indicated that the role of carbon source in the biosynthesis of β-galactosidase may vary and depend on the microorganisms tested.

As a trial to optimize the composition of lactose medium which was proved to be suitable for enzymatic production by *Bacillus* isolate (isolateNo. 12) a series of experiments were conducted.

Different carbon sources were tried to study their suitability for enzymatic production by *Bacillus* isolate (isolate No.12).Carbon concentration of each source was used as that of basal medium (10g/L).Results presented in table 5 clearly indicated that among the tested sources lactose was found to support better yields of enzymatic activity (extracellular and intracellular) as compared to other carbon sources.

Results also showed that the other carbon sources gave varying amounts of enzymatic activity in both extracellular and intracellular enzyme and also enzymatic productivity. The glucose gave the lowest activity and productivity of the test enzyme.

This result was almost similar with Hsu *et al.*, (2005) who found that the final viable population of *B. longum* CCRC 15708 was higher in cultures containing either lactose as the sole carbon source with the highest β-galactosidase activity detected with lactose followed by galactose and the lowest activity with glucose as the carbon source.

Table 5: Effect of different carbon sources on the production of enzyme by bacterial isolate No.12.

Source of carbon	Extracellular enzyme			Intracellular enzyme			Cells fresh weight (mg/ml)
	Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	
Lactose (Control)	390	1497	0.260	1384	1689	0.819	33
Sucrose	206	1000	0.138	400	1128	0.354	12
Glucose	100	600	0.096	278	760	0.157	2
Fructose	208	987	0.104	754	1027	0.371	9
Starch	252	1340	0.155	396	1490	0.563	19
Galactose	116	1011	0.107	481	1077	0.260	10
Dextrane	250	1318	0.125	810	1425	0.400	14
inositol	140	598	0.139	400	890	0.449	17

* Lactose was used as a Control.

-Effect Of Nitrogen Sources On The Enzyme Production:

In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins, and cell wall components. Nitrogen sources may affect microbial biosynthesis of β-galactosidase (Shaikh *et al.*, 1997, Hsu et al., 2005).

Twelve different nitrogen sources (six organic and six inorganic) were used to explore the best ones giving maximal enzymatic production by *Bacillus* isolate (isolate No.12). Each nitrogen source was added to the basal medium in concentration (5 gm/L).

Data in table 6, showed clearly the enzymatic production by bacterial isolate (isolate No.12), greatly affected by the kind of nitrogen source. Results revealed that the use of yeast extract as sole source of nitrogen gave the highest values of enzymatic activity as compared to other inorganic and organic nitrogen sources followed by beef extract, casein hydrolyzed and peptone. While, there were no growth observed with Sodium nitrite, ammonium sulphate and ammonium chloride.

Table 6: Effect of different Nitrogen sources on the production of enzyme by bacterial isolate No.12.

Nitrogen source		Extracellular enzyme			intracellular enzyme			Cells fresh weight (mg/ml)
		Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	Activity (U/ml)	Protein assay (mg/ml)	Specific activity (u/mg)	
Organic source	Yeast extract	410	1626	0.252	1390	1712	0.811	32
	Peptone	292	1371	0.212	760	1432	0.530	10
	Casein	92	427	0.192	150	505	0.215	2
	Casein hydrolysat	308	1452	0.212	806	1502	0.536	12
	Glycine	80	444	0.180	136	455	0.198	8
	Beef extract	360	1586	0.232	870	1665	0.569	20
Inorganic source	Potassium nitrate	58	123	0.089	90	134	0.179	5
	Sodium nitrite	0	0	0	0	0	0	0
	Ammonium sulphate	0	0	0	0	0	0	0
	Ammonium nitrate	116	586	0.170	215	665	0.230	8
	Ammonium chloride	0	0	0	0	0	0	0
	Ammonium sulphate	82	346	0.121	100	543	0.134	1

*yeast extract used as a control

Hsu et al. (2005) reported that yeast extract necessary for β-galactosidase production, while casein, peptone and beef extract repressed β-galactosidase formation. However, other works reported that better β-galactosidase synthesis in the presence of nitrogen sources (Konsoula & Liakopoulou-Kyriakides, 2007) (Nizamuddin *et al.*, 2008).

Conclusion:

Local bacterial isolate (isolate No.12) was screened and identified as *Bacillus subtilis* from agricultural soil and exhibited higher β-galactosidase activity. Lactose 5g/L, yeast extract 10g/L, pH of the medium 7.0 and the incubation temperature at 30°C for 48 hours was the most suitable conditions for the production of β-galactosidase. This suggests that *Bacillus subtilis* (isolate No.12) can be a potential producer of β-galactosidase which could find applications in industry and biotechnology. Additional investigations should be performed to purify this enzyme of this bacterial strain in order to establish its physio-chemical properties with special interest in optimizing its activity.

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