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Isolation and identification of a new yeast isolate with high beta-galactosidase activity from Syrian dairy products

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Abstract

Beta-galactosidase (EC 3.2.1.23), has tremendous potential in research and application in various fields like food, bioremediation, biosensor, diagnosis and treatment of disorders. The main objective of this study was to isolate and select some yeast strains able to produce β-galactosidase from locally dairy products, the high producer strain coded DIYS 11 was belong to *Kluyveromyces marxianus* according to molecular method based on the sequence of the internal transcribed spacer (ITS) rDNA gene, and produced 338 U/min/cell estimated by the use of ONPG as substrate. The effect of six different extraction methods, isoamyl alcohol, acetone, SDS-chloroform, liquid nitrogen, glass beads and stainless beads on enzyme activity from this organism was studied. Our results showed the highest enzyme activity was obtained using isoamyl alcohol and reached 554 U/min/cell after nine minutes incubation with the substrate.

Introduction

Lactose is present in the milk of most at approximately 4.8% (w/v), it is the major carbohydrate in bovine milk and whey (Matioli *et al*., 2003). β-galactosidase (EC 3.2.1.23) hydrolyse the lactose to its two sugars component glucose and galactose at (4 to 40ºC) instead of using acids and high temperature (150ºC) (Gekas and Leiva, 1985). The best process to food industry is the enzymatic due to the less quality losses caused by it, which make this enzyme very important to reduce the amount of lactose in milk and milk derived products especially for people who cannot digest lactose because they lake lactase in their intestine those people called lactose intolerance (Dagbagli and Goksungur, 2008). In addition using β-galactosidase in ice cream and condensed dairy products help to avoid lactose crystallization (Kumari *et al*., 2011a), and the sweet syrup produced by lactose conversion through hydrolysis by β-galactosidase can be used in dairy, baking confectionary and soft drink industries (Panesar *et al*., 2010), another application of β-galactosidase enzyme is the production of galacto-oligosaccarides (Boon *et al*., 2000; Albyrak and Yang, 2002).

There are many microbial sources of β-galactosidase but not all of them are taken or recommended as safe for food use. Yeasts have been considered the predominant microbial enzyme source for food applications including lactase. The activity of enzymes is influenced by the type of strains and the growth medium composition and both *K. fragilis* and *K. lactis* have been the most widely investigated (Dagbagli and Goksungur, 2008) they are now preferential sources for the enzyme production.

B-galactosidase is an intracellular enzyme, which should be released sufficiently from cells using simple, rapid and effective methods to allow small molecules such as substrates or products to cross cells freely and the use of whole cells as a source of β-galactosidase can be used as an important tool in the biotransformation process to be an inexpensive alternative to purified enzyme (Kumari *et al.*, 2011b). Therefore, different methods have been applied to increase their permeability of microbial cell walls (Panesar *et al.*, 2006).

Several workers have reported on the release of β-gal through permeabilization of microbial cells by organic solvents (Numanoglu and Sungur, 2004; Panesar *et al*., 2007; Park *et al*., 2007). Mechanical methods such as sonication, high-pressure homogenizer or bead mills have been traditionally used for the disruption of microbial cells (Geciova *et al.*, 2000). The method of choice should be robust enough to disrupt cell membranes efficiently but gentle enough to preserve enzyme activity (Numanoglu and Sungur, 2004).

Identification and characterization of yeast species have been traditionally based on morphological traits and especially on their physiological capabilities (Kreger-Van Rij, 1984; Barnett *et al.*, 1990). These characteristics are

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strongly influenced by culture conditions and can give uncertain results (Yamamoto *et al*., 1991). The conventional methodology for yeast identification requires evaluation of some 60-90 tests, and the process is complex, laborious and time consuming (Deák, 1995; Deák and Beuchat, 1996).

Recent progress in molecular biology has led to the development of new techniques for yeast identification. The polymerase chain reaction (PCR) amplification of specific sequences for the identification of organisms has become common because of the relative easiness of manipulation and the high reproducibility (Guillamón *et al.*, 1998). Currently, sequence analysis can be applied to differentiate closely related species. Thus, yeast taxonomy can take advantage of molecular methods, especially sequence analysis of the rDNA loci, length polymorphism at the locus ITS1-5.8S-ITS2 (Esteve-Zarzoso *et al.*, 1999; Souza-Liberal *et al*., 2005) and can further classify strains within species (Schuller *et al*., 2004).

The aim of this study is to select a new isolate capable of β-galactosidase production from local dairies and test different disintegration methods on enzyme activity then classify the selected yeast according to DNA based method.

Materials and Methods

Sampling

Dairy product samples including cheese, fresh milk, yogurt, labneh and whey were collected from local markets.

Organisms and culture conditions

Thirty four isolates of yeast isolated from dairy samples were plated on nutrient agar plates. The isolates were purified in petri dishes containing (2% Lactose, 0.5% Yeast Extract, 1% Bacteriological Peptone, 2% Agar, 0.01% Chloramphenicol), and were maintained in slants containing YPDA (1% Yeast Extract, 2% Bacteriological Peptone, 2% Dextrose, 2% Agar, 0.01% Chloramphenicol). The pH was adjusted to 7.0 prior to autoclaving using NaOH or HCl.

Measurement of enzyme activity

ONPG assay was applied for the calculation of enzyme activity in yeast strains (Ortho-nitro phenol B-D- Galactopyranoside, Merk). The method described by Yeast Protocols Handbook (2009) Liquid culture assay using ONPG as substrate. Overnight culture of yeast was prepared in 5 ml liquid selection medium containing (0.5% yeast extract, 1% peptone, 2% lactose, pH = 6-7). On the day of experiment the overnight culture was vortexed for 1 min. and 2 ml were transferred to 8 ml of PYD (1% Yeast Extract, 2% Peptone, 2% Dextrose) , and incubated at 30°C for 3 h with shaking 250 rpm until the cells are in the mid-log phase, OD600 was read using (Hitachi U-2900 spectrophotometer-Japan). After incubation the culture was collected by centrifugation and resuspended in Z buffer, via three freeze/thaw cycles the cell extract were obtained.

After adding 0.7 ml of Z buffer ($Na_2HPO_4.7H_2O$, 60 mM $NaH_2PO_4.H_2O$, 40 mM KCl, 10 mM $MgSO_4.7H_2O$, 1 mM pH = 7) containing β-mercaptoethanol, 160 µl of ONPG (4 mg /1 ml Z buffer) was added and when the yellow color developed, 0.4 ml of Na_2CO_3 (1M) was added to stop the reaction. By centrifugation at 13000 rpm for 5 min, the cell debris was removed and OD was recorded at 420 nm. 1 unit of β-galactosidase is defined as the amount which hydrolyzes 1 µmol of ONPG to O-nitrophenol and D-galactose per min per cell (Miller, 1972; Miller, 1992): β-galactosidase units = 1000 x OD420 / (t x V x OD600).

Cell disintegration

Since β-galactosidase enzyme from *K. marxianus* is an intracellular enzyme, one of the major steps in effective production of this enzyme is its release in sufficient quantities from cells. However, a major drawback is the poor permeability of cell wall membrane (Prasad *et al*., 2013). Therefore, different chemical and mechanical methods were used to permeabilize and/or disrupt *K. marxianus* cells prior to the assay of intracellular β-galactosidase. Hence, the effect of these methods on enzyme activity was determined:

Isoamyl alcohol

0.1 ml of resuspended cells in Z buffer (pH = 7) was mixed with 0.5 ml isoamyl alcohol and the mixture was shaken for 30 min at 30°C to make the cell envelopes permeable and used for the enzyme assay.

Sodium dodecyl sulfate (SDS) – chloroform treatment

Permeabilization of cell membrane was carried out by vortexing 0.1 ml of the cell suspension in the presence of 100 µl chloroform and 50 µl 0.1% SDS solution for 30 min at room temperature (Mahoney *et al*., 1975).

Acetone

10 µl of acetone was added to 100 µl resuspended

cells in Z buffer and incubated for 1 hour with shaking.

Vortexing cells with glass beads

Cell suspensions were vortexed at 4ºC with glass beads (1 mm diameter) in a 30 ml glass tube. Vortexing continued for 10 min and the resulting mixture was centrifuged at 4000 x g. The supernatant was used for the enzyme assay (Song and Jacques, 1997).

Vortexing cells with stainless beads

Cell suspensions were vortexed with stainless beads (1 mm diameter) for 10 min.

Liquid nitrogen

Three times repeat, 0.1 ml of Cell suspensions were frozen in liquid nitrogen for 1 min. then transfer to 37ºC water bath (Yeast Protocol Handbook, 2009).

Genomic DNA isolation

According to Bakri *et al*. (2009), total genomic DNA of selected isolates was extracted from 24 h growing cultures medium (YM) containing per gram: yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1%, 1.5 ml of cultured cells was collected by centrifugation at 14 000 rpm for 5 minutes. Cells were washed with distilled water and digested in 750 µl enzymatic lysis solution (10 µl of proteinase k 20 mg/ml, 2% SDS, 1% 2-mercaptoethanol, 1% CTAB and 10 mM EDTA in 50 mM Tris pH 8 buffer) and incubated for 30 minutes at 60ºC. The lysate was extracted three times repeating of freezing (-80ºC/10 min.), thawing (60ºC/10 min.) then vortexing one minute after adding 2 sterile glass beads The extract was purified by adding 1/10 of the volume of sodium acetate 3 M and 1 ml ethanol. The mix was vortexed and placed for 15 minutes on ice. Sediment high molecular weight DNA was obtained after 5 minutes of centrifugation at 13 000 rpm washed with 70% ethanol and air-dried. The final DNA pellet was dissolved in 50 µl hydration solution and stored at -20ºC. DNA concentration was estimated by measuring the absorbance at 260 nm. The quality of the isolated genomic DNA was calculated by the ratio OD 260 nm/OD 280 nm.

PCR amplification of the 5.8 S rDNA

PCR was used to amplify 5.8 S rDNA gene of β-galactosidase producing isolate. Primers used for PCR and DNA sequencing are ITS1 (5' -TCC GTA GGT GAACCTGCG G-3') and ITS4 (5' -TCC TCC GCT TATTGA TATGC-3'). The standardized PCR conditions were as follows: one cycle of denaturation at 95ºC for 5 minutes followed by 25 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 2 minutes, and one cycle of extension at 72ºC for 10 minutes. PCR products were visualized by electrophoresis in 1% (w/v) agarose gel stained by ethidium bromide, and photographed under UV light. Fragment sizes were estimated by comparison against a DNA standard (100-bp ladder, Fermentase).

5.8 S DNA sequencing

PCR amplicons were purified enzymatically using QIAquik Gel Extraction kit (QIAGEN, Cat. No. 28704), then DNA fragments were tagged (labeled) with Big Dye® terminator cycle sequencing kit (Applied Biosystems). Sequencing reactions were carried out on an ABI 310 genetic analyzer (Applied Biosystem, Department of Molecular Biology and Biotechnology, AECS), and subjected to a BLAST search against the full EMBL/GenBank database available at NCBI public database (*http:// www. ncbi.nlm.-nih.gov*). The sequences obtained were approximately 550 bp Length). All chemicals used in this study were of analytical grade.

Results and Discussions

Isolation and evaluation of highly β-galactosidase yeast producers

To obtain the higher B-galactosidase producing *Kluyveromyces* spp. isolates, different dairy industry samples were used. The best way for the obtaining of yeasts which produced β-galactosidase was isolation by culturing samples into medium containing lactose as the only carbon source (Dagbagli and Goksungur, 2008). The selected yeast isolates were conducted to β-galactosidase assay for selection of the highest enzyme activity according to Yeast Protocol Handbook procedures (liquid culture assay using ONPG as substrate).

In this study, 34 different yeast strains were isolated from local dairy products (Dairy Industry Yeast Syrian) and among them, the DIYS 11strain was found to have the highest enzyme activity 335 units, also the DIYS 10 strain and DIYS 2 strain were also showed high enzyme activity, 307 and 291 unit/ min/cell, respectively. Table (1): shows the results of enzyme units for the best 12 isolates.

Cell disintegration

Cell disintegration is an important procedure applied in the production of intracellular β-galactosidase enzyme from *K. marxianus*. Different mechanical and chemical disruption methods were

Table 1. β-galactosidase activity in the best 12 yeast isolates

Isolate No.	Beta-galactosidase activity (U/min/cell)
DIYS 11	**335**
DIYS 12	307
DIYS 2	291
DIYS 18	207
DIYS 4	196
DIYS 19	191
DIYS 23	138
DIYS 1	112
DIYS 26	98
DIYS 22	68
DIYS 8	59
DIYS 24	47

The specific β-galactosidase activity was measured using ONPG and calculated as follows: Units = 1000 *OD420 / volume (1 ml) *time (min) *OD 600.
We select the strain DISY 11 for further study.

Table 2. Effects of chemical and physical cell disintegration methods on the β-galactosidase activity

	Chemical methods			Physical method		
	Isoamyl alcohol	Chloroform SDS 0.1%	acetone	Liquid nitrogen	Glass beads	Stainless still beads
B-gal activity U/min/cell	**554**	257	294	337	302	464

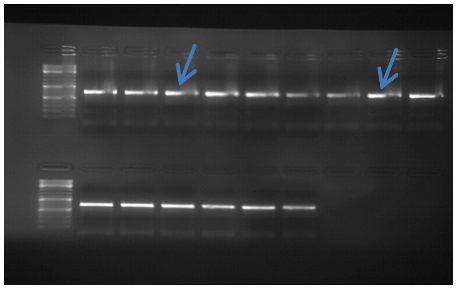

Figure 1. A PCR of 5.8S rDNA fragments of yeasts using ITS1 and ITS4 primers

Arrows indicate to DIYS 11. DIYS 11: *Kluyveromyces marxianus* 99%

AGCCCTGCTCACGCAGATGGCAACGGCTAGCCACTTTCAAGTTAACCCGAGACGAGTATCACTCACTACC
AAACCCAAAGGTTTGAGAGAGAAATGACGCTCAAACAGGCATGCCCCCTGGAATACCAGAGGGCGCAAT
GTGCGTTCAAAGATTTGATGATTCACGAAAATCTGCAATTCACAATACATATCGCAATTCGCTGCGTTCTT
CATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTGAATATTAAATTTTATAGTATAAT

applied to the cell mass before ONPG assay. The results indicated that the activity of β-Galactosidase enzyme is dependent on the cell disintegration method used (Table 2).

Our findings agree with those of Dagbagli and Goksungur (2008) who found that isoamyl alcohol was more effective than glass beads, sonication, liquid nitrogen triton X-100, and SDS treatment for the release of β-galactosidase from *K. lactis* NRRL Y-8279. However our results are contrary to the finding by Gupte and Nair (2010) who concluded that chloroform-SDS was more effective than isoamyl alcohol to release the enzyme from *K. marxianus* NCIM 3551. Several studies have been reported on the permeabilization of microbial cells by organic solvents (Park *et al*., 2007; Panesar *et al.*, 2007), they found that the performance of those solvents was dependent on the incubation time and temperature, and cells and solvents concentration. Kumari *et al*. (2011b) used mixture of chemicals to increase the cell permeability, they used different partners: n-butanol, n-propanol, iso-propanol, acetone, toluene with ethanol and toluene-alcohol mixture gave the best result.

The use of permeabilized cells can help to overcome the problems/costs associated with enzyme extraction and purification from yeast cells and in the development of a low-cost technology for β-galactosidase production (Kumari *et al*., 2011b), however the enzyme extraction method need to be adapted to the strain used (Prasad *et al*., 2013).

Identification of DIYS11isolate

Generally a region of the rDNA gene repeat unit, which includes two non-coding regions designated as the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rDNA gene was amplified, this analysis pattern allows the shortest identification time and relies on a published database that includes most common food-borne yeast species. Identification of DIYS11 was done using 5.8S DNA gene sequences. The nucleotide BLAST similarity search analysis based 5.8S DNA gene sequence revealed that isolate DIYS11 belong to the genus Kluyveromyces. The closest phylogenetic neighbor according to the 5.8S gene sequence data for DIYS11 isolate was Kluyveromyces marxianus with 99% of homology (Figure 1).

Previous results have demonstrated that the complex ITS (internal transcribed spacer) regions (non-coding and variable) and the 5.8S rDNA gene (coding and conserved) are useful since they exhibit far greater interspecific differences than the 18S and 25S rRNA genes (Kurtzman, 1993; Cai *et al.*, 1996; James *et al.*, 1996). There are many studies have used *Kluyveromyces marxianus* to produce lactase for several applications (Brady *et al*.,1995; Bacci *et al.*,1996; Martins *et al*.,2002).

Conclusion

In the present study, the analysis of 5.8S gene sequencing has allowed the identification of β-galactosidase producing organism, DIYS11. The closest phylogenetic neighbor according to 5.8S gene sequence data for DIYS11 isolate was *Kuyveromyces marxianus.* The results indicated that β-galactosidase activity was 554 U/min /cell using Isoamyl alcohol as disintegration method. B - Galactosidase production by *Kluyveromyces marxianus* DIYS11should be optimized for industrial production for further technological applications.

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