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Identification of ß-galactosidase producing fungi isolated from whey and traditional cheese, and investigating the activity of enzyme from the selected isolate

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ABSTRACT

Background and Objective: ß-galactosidases are enzymes of the glycoside hydrolase family (EC 3.2.1.23) that catalyze the hydrolysis of some disaccharides with wide applications in the food industry. Also, some ß-galactosidases have transgalactosylation activity. This study aimed to isolate ß-galactosidase-producing fungi from several traditional cheese and whey samples and to determine the enzymatic activity under the various conditions (temperature, time, pH, and different cations).

Materials and Methods: Yeasts and molds were isolated and screened for the ability to produce ß-galactosidases by a chromogenic test containing 5-bromo-4-chloro-3-indolyl-Beta-D-galacto-pyranoside (X-gal) in the media. After screening, the strongest isolated molds and yeasts were identified genotypically by ITS rRNA sequencing. Then, the hydrolytic activity of crude ß-galactosidase was evaluated using ortho-Nitrophenyl-β-galactoside (ONPG) as the substrate under different conditions (different levels of temperature, time, pH, and various cations Mn^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}). Finally, the possibility of transgalactosylation activity of the selected enzyme was investigated.

Results: The results of PCR product sequencing resulted in the identification of isolates MM24, MT12, and MW14 as *Kluyveromyces lactis* H1-3 (99.57%), *Kluyveromyces lactis* E3 (97.53%), and *Penicillium brevicompactum* (100%), respectively. Investigating the activity of ß-galactosidase produced by MM24, MT12, and MW14 isolates showed a higher activity for the crude enzyme from *K. lactis* MM24. Moreover, the highest activity was found at 37°C, pH 7, and 30 min reaction time. Different cations did not significantly affect the enzymatic activity at 0.1 and 1mM, however it was completely inhibited against Cu^{+2} and Zn^{+2} at 10 mM. Furthermore, 10 mM Mn^{+2} and Ca^{+2} inhibited enzymatic activity by about 80% and 65%, respectively. High-performance liquid chromatography (HPLC) analysis confirmed ß-galactosidase activity on lactose, whereas no transgalactosylation activity was observed in this study.

Conclusion: Overall, *K. lactis* MM24 isolated in the current study can be considered as a new isolate for ß-galactosidase production.

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شناسایی قارچ‌های تولیدکننده بتاگالاکتوزیداز جداسازی شده از آب پنیر و پنیر سنتی و بررسی فعالیت آنزیم حاصل از جدایه منتخب

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واژه‌های کلیدی:
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قارچ
شناسایی مولکولی
فعالیت آنزیمی

چکیده

سابقه و هدف: بتا-گالاکتوزیدازها آنزیم‌هایی از خانواده گلیکوزید هیدرولاز (EC 3.2.1.23) هستند که هیدرولیز برخی دی ساکاریدها را کاتالیز نموده و کاربردهای گسترده‌ای در صنایع غذایی دارند. همچنین برخی بتا-گالاکتوزیدازها واکنش ترانس-گالاکتوزیلاسیون را کاتالیز می‌کنند. این مطالعه باهدف جداسازی قارچ‌های مولد بتا-گالاکتوزیداز از چند نمونه پنیر سنتی و آب پنیر و تعیین فعالیت آنزیمی تحت شرایط مختلف (دما، زمان، pH و کاتیون‌های مختلف) انجام شد.

مواد و روش‌ها: ابتدا مخمرها و کپک‌ها جداسازی و از نظر توانایی تولید بتا-گالاکتوزیداز با استفاده از محیط کشت کروموژنیک حاوی 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal) غربالگری شدند. پس از غربالگری، شناسایی ژنوتیپی قوی‌ترین کپک‌ها و مخمرهای جدا شده با توالی‌یابی قطعه‌ای از ژن rDNA ITS انجام گرفت. سپس فعالیت هیدرولیزی محلول خام آنزیم بتا-گالاکتوزیداز به‌دست‌آمده از جدایه های منتخب با استفاده از ortho-Nitrophenyl-b-galactoside (ONPG) به‌عنوان سوبسترا در شرایط مختلف (سطوح مختلف دما، زمان، pH و کاتیون‌های مختلف (Mn^{2+}، Mg^{2+}، Zn^{2+}، Cu^{2+}) ارزیابی شد. در نهایت، فعالیت ترانس-گالاکتوزیلاسیون محلول خام آنزیمی منتخب مورد بررسی قرار گرفت.

یافته‌ها: نتیجه توالی‌یابی محصول PCR منجر به شناسایی ایزوله‌های MM24، MT12 و MW14 به ترتیب به‌عنوان *Kluyveromyces lactis* H1-3 (99.57%)، *Kluyveromyces lactis* E3 (97.53%) و *Penicillium (brevicom)* (100%) شد. بررسی فعالیت آنزیم‌های بتاگالاکتوزیداز تولید شده توسط جدایه های MM24، MT12 و MW14، فعالیت بالاتری را برای آنزیم خام به‌دست‌آمده از *K. lactis* MM24 نشان داد. همچنین بیشترین فعالیت در دمای ۳۷ درجه سانتی‌گراد، pH ۷ و زمان واکنش ۳۰ دقیقه مشاهده شد. کاتیون‌های مختلف بر فعالیت آنزیمی در غلظت‌های ۰/۱ و ۱ میلی‌مولار تاثیری نداشتند، بااین‌حال فعالیت آنزیم به‌طور کامل در برابر Cu^{2+} و Zn^{2+} در غلظت ۱۰ میلی مولار مهار شد. علاوه بر این در غلظت ۱۰ میلی‌مولار، Mn^{+2} و Ca^{+2} فعالیت آنزیمی را به ترتیب حدود ۸۰٪ و ۶۵٪ مهار کردند. نتایج به‌دست‌آمده از

کروماتوگرافی مایع با کارایی بالا (HPLC) فعالیت هیدرولیتیک آنزیم خام منتخب را روی لاکتوز تأیید نمود. بااین‌حال، فعالیت ترانس گالاکتوزیلاسیون در این مطالعه مشاهده نشد.

نتیجه‌گیری: بر اساس یافته‌های پژوهش حاضر، *K. lactis* MM24 جداسازی شده در پژوهش حاضر می‌تواند به‌عنوان سویه جدید مولد بتا-گالاتوزیداز در نظر گرفته شود.

استناد: یعقوبی، فروغ؛ مویدی، علی؛ خمیری، مرتضی؛ صادقی، علیرضا. (۱۴۰۳). شناسایی قارچ‌های تولیدکننده بتاگالاکتوزیداز جداسازی شده از آب پنیر و پنیر سنتی و بررسی فعالیت آنزیم حاصل از جدایه منتخب. *فرآوری و نگهداری مواد غذایی*، ۱۶ (۲)، ۱۰۲-۸۷.

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Introduction

ß-galactosidases (EC 3.2.1.23), mostly known as lactases, hydrolyze ß-galactosides (e.g. lactose) into monosaccharides (e.g. galacose and glucose). They are extensively used in the dairy industry with the aim of reducing the lactose content and crystallization and increasing sweetness and lactose solubility in dairy products. In addition, it allows the production of lactose-free products suitable for lactose-intolerant consumers. ß-galactosidases are also used to valorize cheese whey to valuable derivatives on a commercial scale [1, 2, 3]. Furthermore, some ß-galactosidases have transgalactosylation activity when a sugar moiety acts as an acceptor that make it possible to synthesize some prebiotics such as galactooligosaccharides (GOS), lactosucrose and lactulose [2, 4].

ß-galactosidases exist in a large variety of organisms such as animals, plants, and microorganisms. Because of high yield and simple handling, extraction of ß-galactosidase from microbial sources is preferred at commercial scale. The most generally used sources for ß-galactosidase synthesis are *Aspergillus niger*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, and *Bacillus subtilis*. Also, enzymes from the microbial sources have higher stability under operational conditions [1, 5]. The optimal conditions for ß-galactosidase activity depend on the enzyme source. For example, yeast ß-galactosidases work better at pH 6 - 7, while most of bacterial ß-galactosidases displays maximum activity at pH levels range from 2.5 - 5.4 [1]. Therefore, yeast ß-galactosidases are mainly used for the production of lactose-free milk and dairy products, while bacterial ß-galactosidases can be suitable for acidic whey hydrolysis. The search for new sources of ß-galactosidases production is momentous as such enzymes may be more active and efficient than existing samples. This research was performed to isolate and identify ß-galactosidase producing fungi and to investigate the transgalactosylation activity of the selected isolate.

In this study, different yeasts and molds were isolated from traditional cheese and whey samples, and then screened to identify new fungi with potential for ß-galactosidase synthesis. The most promising microorganisms were validated as efficient ß-galactosidase producers under submerged fermentation conditions. Finally, the ß-galactosidase activity assay was determined and the effect of various factors such as temperature, pH, time, and cations on enzymatic activity was investigated.

Materials and Methods

Chemicals

O-nitrophenyl - ß- $_D$- galactopyranoside (ONPG) was obtained from Solarbio (Beijing, China). 5-Bromo-4-chloro-3-indolyl-ß-$_D$-galactopyranoside (X-gal), Culture media such as Potato Dextrose Agar (PDA) and Yeast Extract Glucose Chloramphenicol Agar (YGC), mineral salts and other reagents were purchased from Merck co. (Germany).

Microbial isolation

Eight cheese samples (Lighvan, and white-brined cheese) were collected from local retailers (Gorgan, Iran) and two sweet cheese whey samples from Pegah dairy Co. (Gorgan, Iran), and then transferred to the Lab. Different fungi were isolated according to the method described by Yalkin and Ucar [6] and Budak *et al*. [7]. For this, the inoculated dilutions were spread (0.1 ml) in duplicate, over the surface of petri dishes containing PDA (for molds) or YGC (for yeast). The cultured plates were then incubated at 28°C for 3 days for yeast and 7 days for molds, respectively. The distinct colonies were studied in terms of morphological characteristics (color, shape, size, and initial microscopic observation), and pure cultures were prepared. Finally, screening was performed on a chromogenic medium containing X-gal.

Screening by Chromogenic test

The chromogenic test was performed in Petri plates containing (% w/v): lactose (2), malt extract (2), agar (2) and peptone (0.1). The sterilized medium was then supplement with 0.5% (v/v) of X-gal solution (20 mg/mL in dimethyl sulfoxide). After

inoculation, the plates were incubated at 25°C (3 days for yeast and 7 days for mold). Formation of blue color on the plate surface was considered a qualitative indicator of the ability to produce ß-galactosidase [2]. Four levels of response were considered for the chromogenic test: (0) no blue color, (1) light blue, (2) blue, and (3) deep blue. The most promising isolates were selected for molecular identification.

Molecular identification of the fungi and yeast isolates

Yeast and fungal isolates were identified by ITS rDNA region sequencing as reported by Songol and Behbahani [8]. Extraction of DNA was performed by using a commercial DNA extraction kit (GeneAll kit, Seoul, Korea) according to the manufacturer's instructions.

The amplification was carried out using ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers [10]. The PCR reaction mixture of 30 µl was prepared by using 15 µl of PCR master mix, 1.5 µl of each primer (0.5 uM), 1 µl of DNA template (200 ng), and 11 µl of sterile Milli-Q water. The samples were amplified using a thermocycler (CG1-96, Corbett, Australia), under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 2 min, extension at 72 °C for 2 min and the final extension at 72 °C for 10 min. Analysis of PCR product was done by electrophoresis by using 1.5% agarose gel containing 1 µl DNA safe stain.

PCR products were sent for sequencing to Gene Researcher Cell Company (Tehran, Iran). By using BLAST search, identification and similarity of the queried sequence were tested. After identity percent checking, the sequenced data was submitted to the National Center for Biotechnology Information (NCBI) Gen Bank to attain accession numbers.

ß-galactosidase production

Fermentation medium was composed of (%w/v): peptone (0.5), lactose (2), yeast extract (0.1), $Na_2HPO_4.12H_2O$ (1), KH_2PO_4 (0.25) and $MgSO_4.7H_2O$ (0.125) [2]. After the inoculation with 2% spore suspension or active yeast cells, the fermentation was performed for 7-8 days at 150 rpm (37°C) for molds and 6 days at 150 rpm for yeast (37°C). Finally, the fermentation liquid was centrifuged for 10 min at 4500 rpm and supernatant was used as crude enzyme solution.

ß-galactosidase activity assay of selected isolate

After obtaining crude enzyme from selected isolates (MW14, MT12, and MM24), the ß-galactosidase activity was assayed using ONPG as substrate. 200 µL of crude enzyme was mixed with 200 µL of ONPG solution (3 mM) previously prepared in sodium phosphate buffer (100 mM) or sodium citrate buffer (50 mM) and incubated at 37°C for 30 min. The reaction was terminated by adding 800 µL of sodium carbonate (100 mM) [2, 10]. The released O-nitrophenol was detected using a spectrophotometer at 415 nm. One unit (U) of the enzyme was defined as the amount of enzyme that liberates 1 µmol of O-nitrophenol from ONPG per minute under the assay conditions.

Effect of different parameters on the enzymatic activity

Effect of time

The activity of ß-galactosidase (from selected isolates) was investigated using ONPG solution in sodium phosphate buffer (pH 7) at 37°C for 30, 50, and 120 minutes. After terminating the reaction, the activity of crude enzyme was determined as described above.

Effect of temperature

In order to determine the effect of temperature, enzymatic activity of ß-galactosidase solution was assayed by ONPG solution in sodium phosphate buffer (pH 7) at various temperatures (25, 30, 37, and 45°C) for 30 minutes. After terminating the reaction, the activity was calculated (as described at 2.6 section).

Effect of pH

The effect of different pH values on the enzymatic activity of the ß-galactosidase was examined using ONPG solution (3 mM), in sodium citrate buffer (pH 3-5), and sodium phosphate buffer (pH 6-8) at 37°C for 30 minutes.

Effect of different cations on enzyme activity

The crude enzyme solutions were assayed with 3M ONPG solution [in 10 mM Bis-Tris (pH 6.5)] in the presence of various cations, including calcium chloride, zinc chloride, manganese sulfate, and copper sulfate with final concentrations of 0.1, 1, and 10 Mm. The same conditions (30°C for 10 minutes) were considered to assay the enzymatic activity of ß-galactosidase without the addition of cations [11].

Verification analysis of ß-galactosidase and trans-galactosylation activity by HPLC

The sugar composition of cheese whey treated with crude enzyme solution was qualitatively analyzed by high-performance liquid chromatography (HPLC) equipped with refractive index detector and analytical column (4.6 mm*250) SPHERISORB NH_2, according to Silverio et al. [2] and Zhou and Chen [12]. A mixture of acetonitrile: water (70:30 v/v) at 1 mL/min was used as the mobile phase and the injection volume was 20 µL. Finally, qualitative analysis was performed by comparing the chromatograms to those of pure standards (lactose, galactose, and Melezitose).

Statistical analysis

In this study, all experiments were performed in at least two replications. a completely randomized design was conducted using SAS software (version 9.1). Mean comparisons were performed using Duncan's multiple range test at a 95% confidence level.

Results and Discussion

Screening for potential strains

In total, 35 distinguished colonies were isolated from cheese and whey samples. The results of initial chromogenic test are presented in Table 1, considering the response levels established beforehand (section 2.3). Among the 35 fungal strains tested, 18 strains could produce ß-galactosidase with varying positive response levels.

Among the molds and yeast isolates, MM25, MW14, MG21, MT12, ML12, MM21, MM23, and MM24 codes exhibited better response, and were regarded as the promising ß-galactosidase producers. In a similar study, Silverio *et al*. [2] used the chromogenic media on 50 fungal strains, of which 12 strains showed high potential (level 3, deep blue). In another study, *Aspergillus lacticoffeatus* showed a positive response to the chromogenic test, indicating the potential to produce ß-galactosidase [13]. The chromogenic test with X-gal has been proposed as a simple and fast assay for measuring the production potential of ß-galactosidase. However, as mentioned before, this assay only provides a qualitative evaluation, and therefore we need to design additional quantitative assessments to confirm the effective production of ß-galactosidase by the yeast and mold strains [2].

Molecular identification of the fungi and yeast isolates

The sequence of the ITS rDNA amplicon (~ 650bp) of the isolates (MM24, MT12, MW14) was compared to those in the NCBI Gen Bank database. Sequence data exhibited 99.57%, 96.53%, and 100% BLAST homology with MM24, MT12, and MW14, respectively (Fig. 1). According to the sequencing results of PCR products, isolates MM24, MT12, and MW14 were identified as *Kluyveromyces lactis* H1-3, *Kluyveromyces lactis* E3, and *Penicillium brevicompactum*, respectively.

Table 1. Evaluation of the ß-galactosidase activity of 35 fungal and yeast isolates on chromogenic medium

Code	Isolate	Level[a]	Code	Isolate	Level[a]	Code	Isolate	Level[a]
MT11	Yeast	0	MW11	Yeast	1	MT14	Mold	0
MT12	Yeast	3	MW12	Yeast	0	MW13	Mold	0
MT13	Yeast	0	MN11	Yeast	1	MW14	Mold	0
MT15	Yeast	0	MN23	Yeast	0	MW15	Mold	0
MG11	Yeast	1	MN24	Yeast	1	MW16	Mold	0
MG12	Yeast	2	MM21	Yeast	3	MW17	Mold	1
MG13	Yeast	0	MM22	Yeast	2	MM25	Mold	3
MG21	Yeast	3	MM23	Yeast	3	MM26	Mold	1
MG22	Yeast	0	MM24	Yeast	3	MN12	Mold	0
ML13	Yeast	0	ML11	Yeast	2	MN13	Mold	0
ML15	Yeast	0	ML12	Yeast	3	MN21	Mold	0
			ML16	Yeast	0	MN22	Mold	1

[a] Level of response: (0) no blue, (1) light blue, (2) blue, (3) deep blue

Similarly, the presence of ß-galactosidase producing *Kluyveromyces* spp. in cheese and whey was confirmed through molecular identification by other researchers. In this term, *K. lactis* has been found to be the most abundant species [14, 15]. In other studies, *K. lactis* and *Penicillium* species such as *P. brevicompactum* as ß-galactosidase producing isolates were isolated from fermented dairy products [2, 7, 16, 17, 18]. Moreover, In the study conducted by Silverio et al. (2018) *P. brevicompactum* had the highest ability to produce ß-galactosidase among identified fungal species [2].

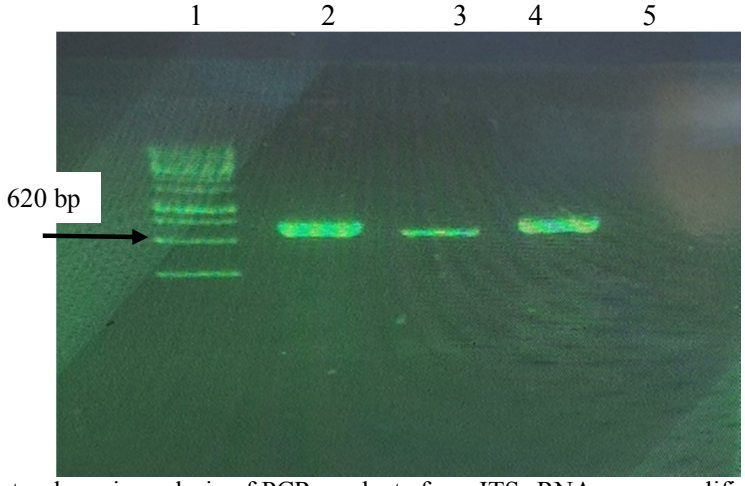

Figure 1. Electrophoresis analysis of PCR products from ITS rRNA gene amplification of three selected yeast and mold isolates on agarose gel (1.5%, w/v). Lane 1: DNA marker; Lane 2: MM24; Lane 3: MT12; Lane 4: MW14; Lane 5: Negative control

Crude ß-galactosidase production by selected strains

The submerged fermentations were performed in a medium containing lactose for 8 days for *Penicillium brevicompactum* (MW14) and 6 days for *K. lactis* (MM24) and *K. lactis* (MT12). The fermentate was centrifuged (4500 rpm) for 10 min and the supernatant was collected and used as the crude enzyme solution.

The activities of crude enzymes from three selected isolates MM24, MT12, and MW14 were tested in the media containing ONPG (3 mM) as the substrate at pH 7 and 4.5 (Fig. 2). According to the results, the MM24 exhibited the highest activity at pH

7 (1.2 U/mL) and 4.5 (0.51 U/mL), whereas the MW14 showed the lowest activity (0.04 U/mL) at pH 4.5. Therefore, crude enzyme from MM24 was selected for further evaluations.

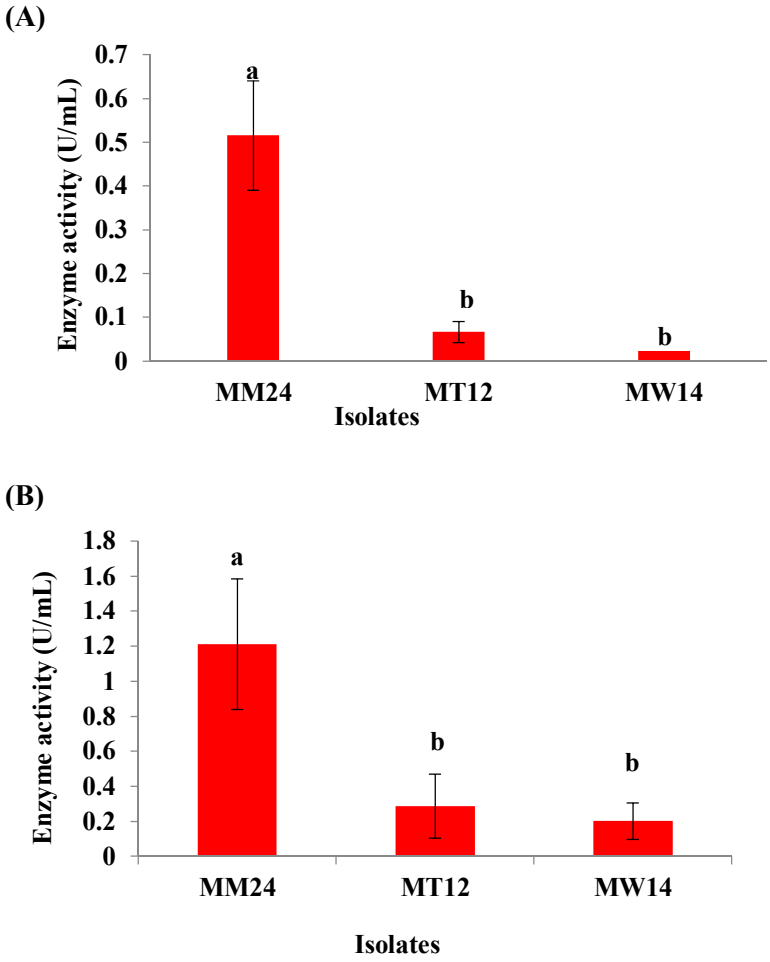

Figure 2. ß -galactosidase activity of three mold and yeast isolates at pH 4.5 (A) and pH 7 (B)

Effect of differenet parameters on the enzymatic activity of crude enzyme from *Kluyveromyces lactis* MM24

Effect of time

The effect of different incubation times on the enzymatic activity of crude enzyme from *K. lactis* MM24 is shown in Fig. 3a. The highest enzymatic activity (0.933 U/mL) was observed at 30 min. With increasing incubation time, the activity of ß-galactosidase significantly decreased, reaching the lowest activity (0.277 U/mL) after 120 min incubation. This finding is consistent with a study conducted by Cardoso *et al*. [13] on ß-galactosidase derived from A. *lacticoffeatus*, where they also observed maximum enzymatic activity after a 30-minute incubation. These finding are also in line with findings reported by other researchers [2, 10, 18].

Effect of temperature

Fig. 3b. presents the effect of temperature on the ß-galactosidase activity from *K. lactis* MM24. The enzymatic activity at 37°C was found to be significantly higher than those measured at 25, 30, and 45 °C and the ß-galactosidase activity decreased when the temperature was more than 37 °C. In a similar study, Ebrahimi and Nejati [19] investigated the effect of temperature on the activity of ß-galactosidase produced by *K.*

marxianus isolated from whey. Their results indicated that 37 °C was more favorable to the ß-galactosidase activity. The results of Nagy *et al*. [18] were also consistent with our results. In their study, the ß-galactosidase activity of *P. chrysogenum* increased as the temperature rose to 30-37°C and significantly decreased when the temperature was more than 37 °C. The highest ß-galactosidase activity for mesophilic organisms has bene reported around 35-37 °C [20].

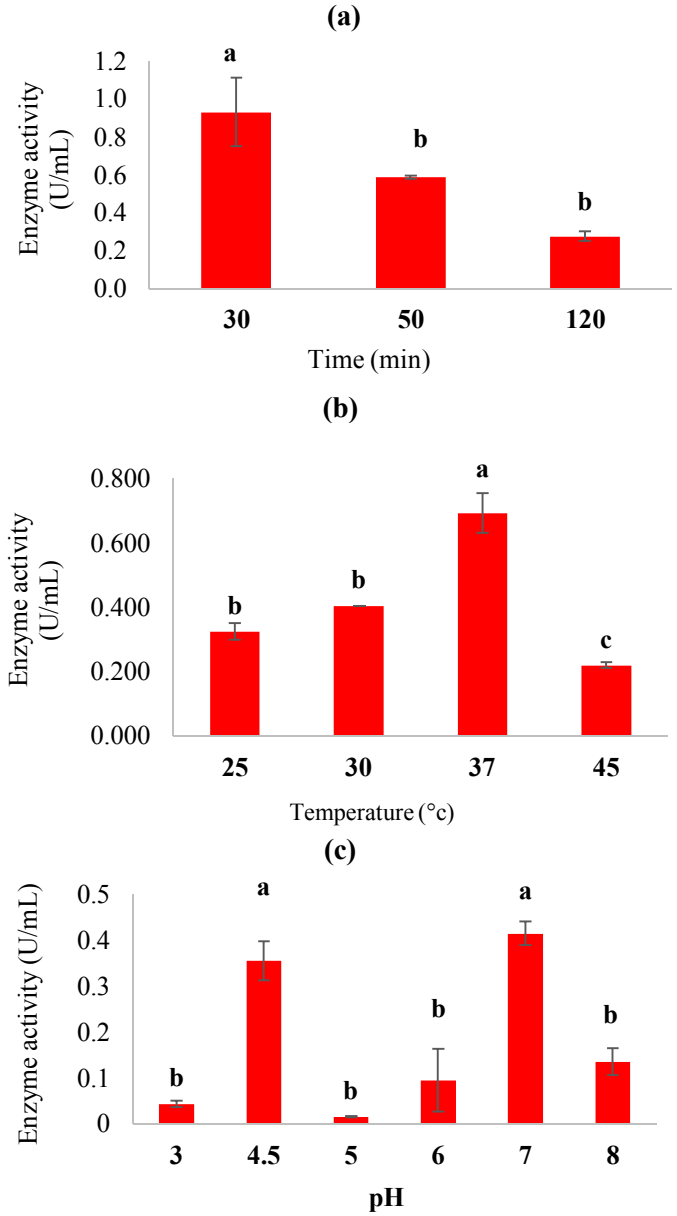

Figure 3. Effect of different levels of time (a), temperature (b), and pH (c) on the activity of crude enzyme from the selected isolate

Effect of pH

The results of ß-galactosidase activity at different pH values are shown in Fig. 3c. The enzymatic activity at pH 7 (0.416 U/mL) and 4.5 (0.36 U/mL) was significantly higher than the activity measured at other pH levels. Meanwhile, no significant effect on the activity was found

between pH 3, 5, 6, and 8. High enzyme activity at two pH levels (4.5 and 7) can be attributed to the presence of isoenzymes in the crude enzyme as described by Silverio *et al.* when studying the activity of enzyme from *A. brasiliensis* [2].

In consistent with our observations, the activity of ß-galactosidases derived from different *K. lactis* strains was significantly higher in the neutral pH range [1, 3, 21]. Zhou and Chen (2001), reported that the optimal pH for the ß-galactosidase activity was reported between 6 and 7, and the lowest activity was observed at pH 5.5 [12]. Moreover, in the study conducted by Pavithra and Thirumagal [20], the highest and the lowest ß-galactosidase activity were found at pH 7 and pH 5 and 9, respectively. In another study, ß-galactosidases derived from *P. chrysogenum* showed an optimal activity in the pH range between 4 and 5, whereas their activity was decreased at lower and higher pH. It was also reported that the decrease was more pronounced for the pH lower than 4 [18]. For ß-galactosidase derived from *A. oryzae*, the optimum activity was found at pH 4.8, almost similar to our observations [22]. Moreover, Carevic *et al*. [23] suggested a pH range of 5 to 7.5 as the optimal conditions for the activity of ß-galactosidase derived lactic acid bacteria. However, they observed the maximum activity at pH 6.8, which agrees with our results. Research by some researchers on the effect of pH on the activity of ß-galactosidase obtained from yeast sources, including strain *k. lactis* has been done. The results show that the optimal pH for ß-galactosidase activity from yeast sources is 6 to 7 and in the neutral range [1, 3].

Effect of concentration of cations

Based on the literature review, various cations can have inhibitory or stimulatory effects on enzymatic activity [20, 24]. In the present study, the ß-galactosidase activity was measured in the presence of four cations: Zn^{2+}, Cu^{2+}, Mn^{2+}, and Ca^{2+}, in the concentration range of 0.1– 10 mM. The effect of cations concentration on the enzymatic activity was compared in Fig. 4. The lowest activity of ß-galactosidase was seen at 10 mM concentration of Zn^{2+} and Cu^{2+} cations, as the enzymatic activity was completely inhibited. This negative effect was followed by Mn^{2+} and Ca^{2+} cations, with a decreased activity of about 80 and 65%, respectively. However, the lower concentrations of cations (0.1 and 1 mM) did not show any significant effects. Therefore, it can be concluded that the ß-galactosidase activity decreased with the increasing concentration of tested cations. This effect can be explained by the competitive mechanism between the cations in the active site of the enzyme and the substrate, which causes the cation to occupy the active site of the enzyme. It is worth noting that the enzyme requires a certain amount of metal ions; however, high level of cations upsets the surface charge balance of the enzyme and thus prevents enzymatic activity. The same results for the activity of ß-galactosidase produced by *Bacillus* strains and *K. lactis* were reported by other researchers [20, 24, 25, 26]. Moreover, the activity of ß-galactosidases derived from *Lactobacillus reuteri* decreased in 10 mM Zn^{2+}, Mg^{2+}, and Ca^{2+} by about 100, 50, and 30%, respectively. However, these cations increased the activity at concentrations around 1 mM [11]. Garman *et al*. reported a significant decrease in enzymatic activity when cations concentration was increased above 50 mM [27]. They did not also observe any significant effect in the concentrations lower than 5mM. Pavithra and Thirumagal, reported that 1 mM concentration of cations had no effect on enzyme activity while if the concentration of cations increased, the activity of the enzyme also decreased [20].

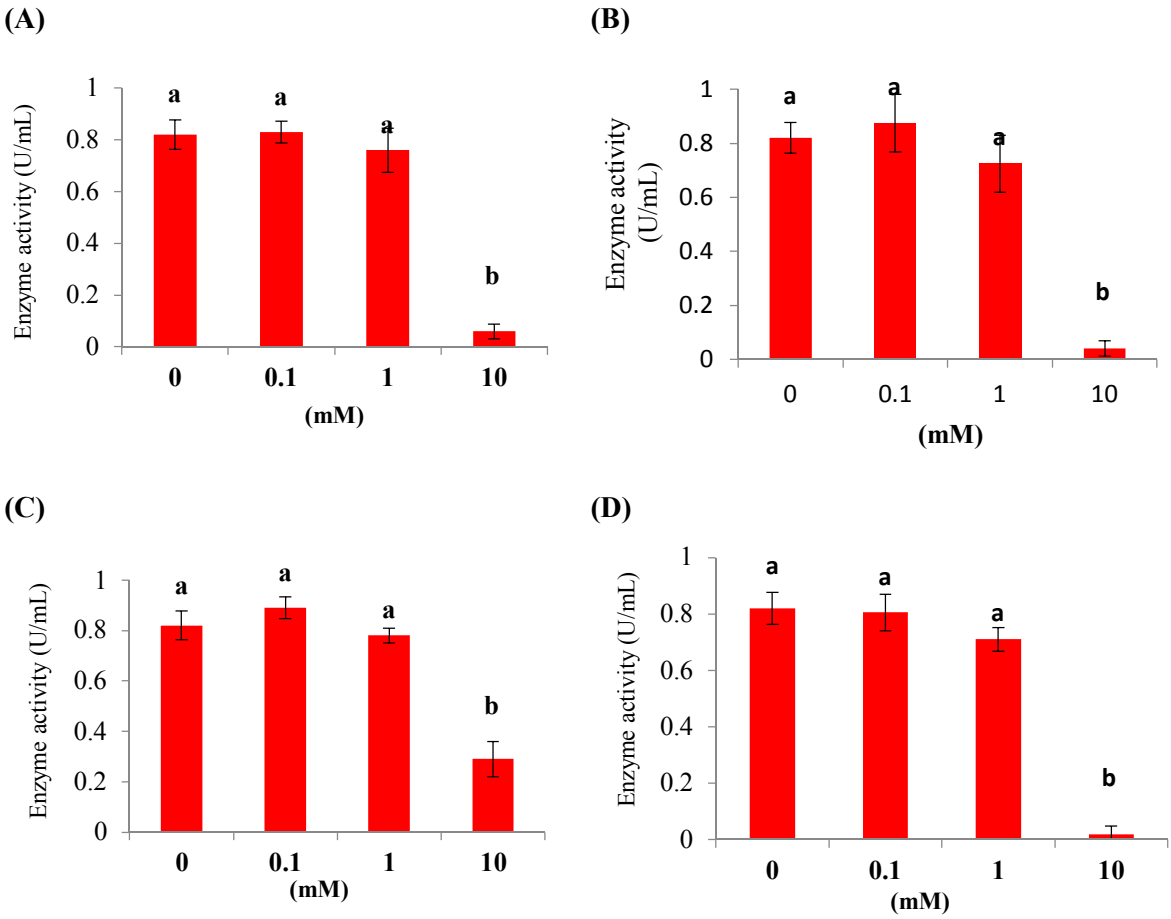

Figure 4. Effects of various cations on ß-galactosidase activity: Mn^{2+} (A), Cu^{2+} (B), Ca^{2+} (C) and Zn^{2+} (D)

HPLC Analysis

The qualitative analysis of hydrolytic activity of crude enzyme on lactose was done using a high-resolution HPLC. The analytical results demonstrated reasonable hydrolysis of lactose, which indicated a high level of ß-galactosidase activity as reflected by formation of galactose in the media (Fig. 5). However, transgalactosylation activity was not observed under the conditions examined here. In a similar study, Zhou and Chen [12] reported positive results of lactose hydrolysis for *K. lactis* ß-galactosidase, while no evidence of transgalactosylation activity was detected by the same strain. In another study on galactooligosaccharides synthesis by *Lactobacillus acidophilus* ß-galactosidase, Carevic *et al.* demonstrated transgalactosylation activity besides lactose hydrolysis [23]. Lactose concentration and enzyme purity can affect the reactions that result in production of galactosyl-oligosaccharides [1, 2].

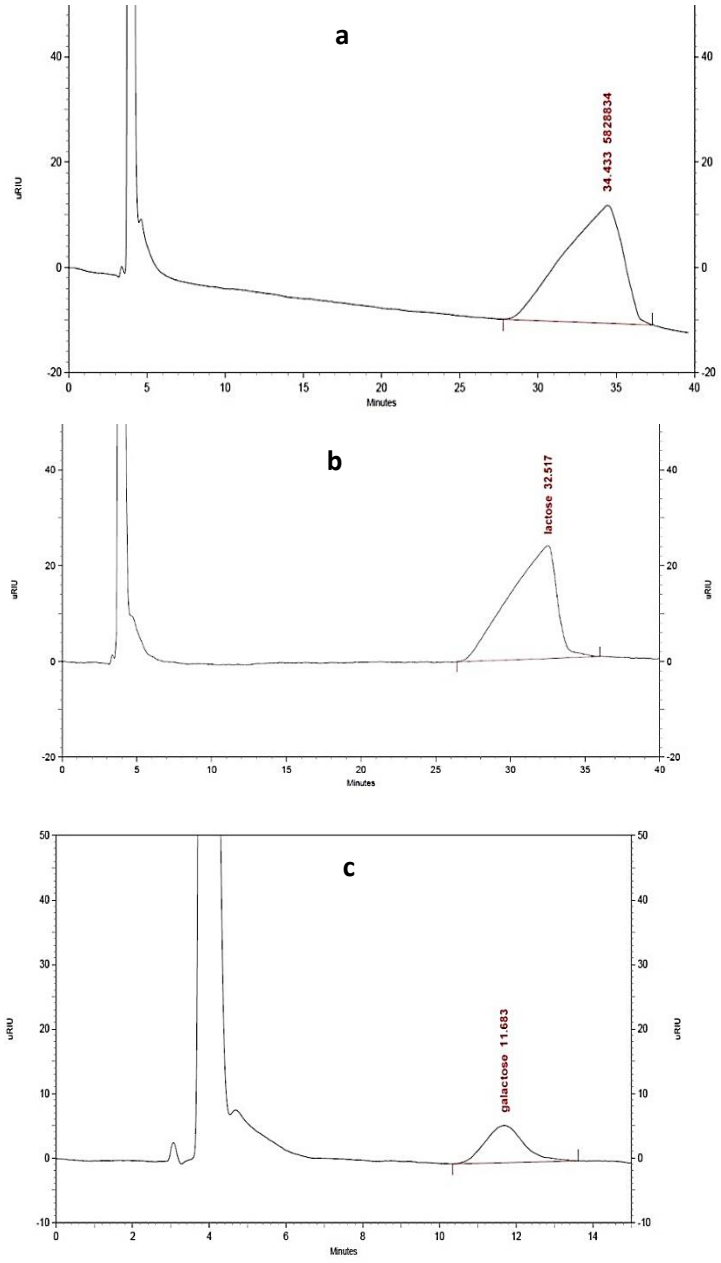

Figure 5. HPLC chromatogram: control lactose solution (a), (b) lactose solution treated with crude enzyme containing lactose (b) and galactose (c).

Conclusion

The present study was performed to isolate new strains of ß-galactosidase-producing fungi and to evaluate the effects of experimental conditions on the enzyme activity. *K. lactis* MM24 was found to be a promising yeast strain for ß-galactosidase production. The optimum conditions for enzyme activity was found at pH 7 (0.42 U/mL) and 37 ^{o}C (0.70 U/mL). Although the crude enzyme from *K. lactis* MM24 displayed reasonable hydrolytic activity on lactose, it did not show transgalactosylation activity under the tested conditions. The investigation of other conditions such as different lactose concentration is recommended for better conclusion on the transgalactosylation activity which is important to synthesize oligosaccharides with prebiotic potential.

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Conflict of Interest
The authors report no conflicts of interest.

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