Research Article

Isil Seyis Bilkay*

Investigation of lactose hydrolysis by crude extract of *Trichoderma viride* ATCC 32098

*Trichoderma viride* ATCC 32098’in kaba ekstraktıyla laktoz hidrolizinin araştırılması

DOI 10.1515/tjb-2016-0309
Received March 14, 2016; accepted October 11, 2016; previously published online March 25, 2017

Abstract

Objective: Aim of this study was to find the optimum pH, temperature and incubation conditions for efficient lactose hydrolysis by beta-galactosidase produced by *Trichoderma viride* ATCC 32098. In addition, enzymatic lactose hydrolysis in milk, whey and lactose solutions were studied and compared.

Material and methods: *Trichoderma viride* ATCC 32098 was incubated at 30°C for 8 days on a rotary shaker. Lactose hydrolysis was calculated based on the amount of glucose measured. Hydrolysis was carried out in lactose solution, milk and whey.

Results: Maximum efficiency was observed at pH 5.0 and at 60°C. Agitation increases lactose hydrolysis slightly. When enzymatic lactose hydrolysis in milk, whey and lactose solutions were studied, it was observed that after 26 h, hydrolysates in lactose solution and whey were almost 100% and hydrolysis in milk was 92%.

Conclusion: It can be concluded that the enzyme produced from *T. viride* ATCC 32098 in this study is a good alternative for use in lactose hydrolysis.

Keywords: Lactose hydrolysis; *Trichoderma*; Beta-galactosidase; Milk; Whey.

Özet

Amaç: Bu çalışmanın amacı, *Trichoderma viride* ATCC 32098’den üretilen beta-galaktosidaz enziminin verimlili

---

*Corresponding author: Isil Seyis Bilkay, Faculty of Science, Department of Biology, Biotechnology Section, Hacettepe University, Beytepe, Ankara 06532, Turkey, e-mail: iseyis@hacettepe.edu.tr

Introduction

Lactose is a substance found in milk and milk products. Milk produced by lactose hydrolysis can either be consumed as milk or used in a variety of products such as ice cream. In addition, as the sweetness of milk increases by lactose hydrolysis, amount of sugar added to the milk or dairy products is decreased, which is important from economical point of view especially in countries where sugar prices are considerably high [1–3].

Lactose intolerance is caused by a deficiency of lactase, which has ability to digest lactose. Almost half of the world population cannot hydrolyze lactose due to lack of this enzyme. To avoid this problem, lactose needs
to be hydrolyzed into simpler sugars such as glucose and galactose [4–6].

On the other hand, whey is the major byproduct waste of dairy industry. In 2014, whey products all over the world were approximately 5.6 million tons. As the amount of whey produced is directly proportional to the increase in cheese production, an increase of cheese production up to 25.3 million tons by 2023 will result in a considerable amount of whey produced. This brings an adverse environmental impact due to its high biochemical oxygen demand (BOD) content and should be treated properly before disposal, in which high treatment costs are encountered. In addition, sugar in whey cannot be utilized when it is treated and disposed. Utilization of such potential products receives more attention when the continuously increasing world population and the limited food reserves especially in some parts of the world are taken into account [7].

Lactose is the major component of whey. Therefore, β-galactosidase (EC 3.2.1.23) is important for the proper utilization of this product by lactose hydrolysis. The whey processed in this way is used in various industries such as bakery, beer and beverage industries. In general, there are several technologies for enzymatic hydrolysis of lactose using lactase [2, 7–11].

Although a variety of microbial enzymes are used for lactose hydrolysis recently, there is still a need for more stable enzymes having a wide area of application. Therefore, in this study Trichoderma viride was studied as an alternative fungal source. In this respect, lactose hydrolysis by β-galactosidase produced from T. viride ATCC 32098 was investigated. First of all, the effect of pH, temperature and incubation conditions on the enzymatic lactose hydrolysis was studied. Following, enzymatic lactose hydrolysis in milk, whey and lactose solutions were studied and compared.

Materials and methods

Microorganisms

Trichoderma viride ATCC 32098 was used for the production of β-galactosidase. Trichoderma viride strain is a filamentous fungus having green conidia [12]. Trichoderma viride ATCC 32098 was maintained on potato dextrose agar slants at 4°C and transferred to fresh slants incubated at 30°C in 3–4 days.

Media and inoculation procedure

Medium described by Fiedurek and Ilczuk [13] was used with some modifications for growth and enzyme production. The composition of this medium was (as g L⁻¹): 10.0 lactose, 1.5 peptone, 1.0 yeast-extract, 1.0 KH₂PO₄, 7.0 (NH₄)₂HPO₄, 1.0 MgSO₄·7H₂O and 0.3 CaCl₂. Enzyme was produced in 50/250 mL flasks. The medium was adjusted to pH 5.0 before autoclaving (121°C, 1.5 atm, 15 min). Trichoderma viride ATCC 32098 cultures were grown on potato-dextrose agar for the preparation of spore suspensions. One mL spore suspensions containing 15 × 10⁶/mL was inoculated into 50 mL growth media. Incubations were carried out at 30°C for 8 days on a rotary shaker (150 rpm).

Assay of lactose hydrolysis

The culture filtrate was centrifuged at 7200 rpm for 15 min and used as the enzyme sample. Lactose hydrolysis was determined with the method described by Park et al. [14]. Lactose (Merck) solution of 10 mL was added to 2.5 mL of enzyme sample in the reaction tubes, and incubated at 50°C for 2 h. The reaction was terminated by boiling the mixture for 10 min. The amount of glucose was measured by Trinder reagent (Sigma). The assay was performed by mixing 50 μL of a sample and 1 mL of Trinder reagent, incubated for 30 min at room temperature and the absorbance at 505 nm (using Jenway 6105 uv/vis spectrophotometer) was read. Sodium-acetate buffer was used as control. The absorbance was related to the concentration of glucose with a standard calibration curve [3].

The percentage of lactose hydrolysis was defined as the ratio between the concentration in glucose produced and the concentration of glucose theoretically produced by 100% hydrolysis of the substrate [15].

Effect of pH and temperature on the enzymatic hydrolysis of lactose

Investigation of the effect of pH on the enzymatic hydrolysis of lactose was carried out in the standard assay mixture except that the reaction pH was changed between 4.0 and 7.0. The effect of temperature on lactose hydrolysis was determined by incubating reaction mixture at 30°C, 40°C, 50°C, 60°C and 70°C.
Effect of incubation condition on the enzymatic hydrolysis of lactose

In order to investigate the effect of agitation and static incubation conditions on the enzymatic hydrolysis of lactose, two parallel experiment sets were used. The incubations were carried out at 30°C for 2 h in both sets, except the first set was agitated at 150 rpm where the second was static.

The enzymatic hydrolysis of lactose in lactose solution, milk and whey

*Trichoderma viride* ATCC 32098 was incubated at 30°C for 8 days on a rotary shaker (150 rpm). The culture was centrifuged at 7200 rpm for 15 min and used as the enzyme sample. Lactose hydrolysis was carried out in lactose solution, milk and whey. Lactose solution (Merck) was prepared in sodium-acetate buffer (pH 5.0, 0.1 M) as 4.7 g/L. Enzyme sample of 10 mL was mixed with 40 mL lactose solution, milk or whey in the reaction tubes and incubated at 50°C. Samples at certain time intervals were taken and analyzed.

Results

In order to find the optimum conditions for lactose hydrolysis, effect of pH, temperature and incubation conditions were studied.

In this respect, first of all, effect of pH on lactose hydrolysis was investigated and it was observed that maximum hydrolysis was at pH 5.0. Hydrolysis rate decreased slightly (5% of the maximum value) with increasing pH up to 7.0 (Figure 1).

Another factor affecting lactose hydrolysis is the incubation temperature. In our study, considerable amount of hydrolysis was observed in the temperature range 40°C–60°C where maximum hydrolysis was observed at 60°C (Figure 2).

When the incubation condition on lactose hydrolysis was investigated it was observed that agitation increased the hydrolysis only 3% (Figure 3).

Lactose hydrolysis of the enzyme produced was determined in lactose solution, milk and whey (Figure 4). It was observed that hydrolysis increased continuously between 2 and 26 h in all of these media. After 10 h lactose hydrolysis was 83% in lactose solution, 77% in whey and 75% in milk.

Finally, time periods at which complete hydrolysis took place was determined. As it can be seen from

Discussion

Lactose hydrolysis can be performed by strong mineral acids or enzyme. In chemical hydrolysis, high temperatures should be maintained as well as high acid concentrations
Isil Seyis Bilkay: Investigation of lactose hydrolysis by crude extract of *Trichoderma viride*

and this method has several disadvantages such as color change, bad odor and unpleasant taste [1, 16]. Therefore, enzymatic hydrolysis is preferred especially when the products are to be used in food or beverage industry. In a previous study, it was observed that *T. viride* ATCC 32098 was an effective lactase producer [17]. In this respect, efficiency of lactose hydrolysis using *T. viride* ATCC 32098 was studied.

Efficient lactose hydrolysis can be achieved by optimizing pH, temperature and incubation conditions. In similar studies carried out with mold based lactases such as *Aspergillus niger* and *Pencillium notatum*, lactose hydrolysis were maximum at acidic pH values, which is parallel with our findings [18–21]. This fact limits the usage of these enzymes in products such as milk that are at higher pH values (pH 7.0) and is considered to be a disadvantage [11]. However, as stated above the hydrolysis rate of enzyme produced in this study is above 50% at pH 7.0, and therefore can be used at neutral pH values and especially in milk.

At the temperatures, which are near to pasteurization where microbial growth is limited, availability of using these enzymes is extremely important [7, 11]. The enzyme produced in this study enables lactose hydrolysis before or during pasteurization together with the elimination of pathogenic bacteria at desired temperatures.

Mould based β-galactosidase are generally effective at 50°C–60°C [18, 19, 21, 22]. In a study carried out by Tanriseven and Dogan [23], it was reported that the enzyme lost its activity at 70°C. In fact, the enzyme produced in our study was still active at 70°C, which can be considered as an advantage. On the other hand, β-galactosidase produced from yeasts denature rapidly above 40°C, therefore they are not preferred for lactose hydrolysis.

Effect of agitation was investigated in our study so as to determine the amount of increase in lactose hydrolysis. Theoretically, increase in hydrolysis is an expected result as the probability of enzyme coming across the substrate is increased with agitation. However, an increase of only 3% was achieved with agitation and it can be concluded that agitation is not feasible when the economics of the process is considered.

Lactose hydrolyzed products attract increasing attention as they have widespread use in industry. In this respect, various technologies have been developed for using β-galactosidase (also known as lactase) in lactose hydrolysis [21, 24]. These technologies are selected based on various factors such as economics of production, storing and marketing requirements depending on the structure of the substrate. The most important factor is the condition at which hydrolysis takes place. Proper selection of the enzyme is important for efficient hydrolysis in milk and whey, as it is the case in all products. In general, for each single process different enzymes are used.

In this respect, this study aimed to find an efficient enzyme that is suitable for use in both milk and whey. Both activity and the stability of the enzyme should be high at different pH values and temperatures.

It should be noted that, 75% hydrolysis in milk with pH 7.0 is a promising result as many other enzymes in literature were not active at these neutral pH values [18–21].

In a previous study, it was concluded that mold based lactases were more suitable for use in whey rather than milk due to the pH values of these products [22]. In addition, it was stated that composition of milk affects the enzyme activity [11]. But, in our study, 75% hydrolysis in milk shows that this enzyme is relatively stable.
As stated above, lactose hydrolyzed products are used in many industrial processes. In the production of some fermentative products, especially in cheese production where the production time is relatively long, a pre-hydrolysis process is needed. In the products produced especially for lactose intolerant people, lactose hydrolysis is extremely important. On the other hand, desired amount of hydrolysis varies depending on the process, as the sweetness increases considerably with hydrolysis, which is not desirable in all products [2, 7, 16].

In a previous study carried out with P. notatum, lactose hydrolysis in whey (pH 4.0, 50°C, containing 5% lactose) was observed to be 98% after 48 h [19]. Similarly with K. lactis, lactose, hydrolysis in whey (pH 7.0, 35°C, containing 5% lactose) was 96% after 48 h [25].

As a result, the enzyme produced from T. viride ATCC 32098 in this study can be a good alternative for use in lactose hydrolysis especially at high temperatures (50°C–60°C). In addition, another advantage of the enzyme is the high lactose hydrolysis rate in milk when compared to the results of similar studies.

References

1. Akgul FB, Demirhan E, Ozbek B. A modelling study on skimmed milk lactose hydrolysis and β-galactosidase stability using three reactor types. Int J Dairy Technol 2012;65:217–31.
2. Uhlig H. Carbohydrate Hydrolyzing Enzymes, Industrial Enzymes and Their Application. New York: John Willey & Sons Inc., 1998:37–137.
3. Obon JM, Castellar MR, Iborra JL, Manjon A. β-galactosidase immobilization for milk lactose hydrolysis: a simple experimental and modeling study of batch and continuous reactors. Biochem Educ 2000;28:164–8.
4. Abbasi S, Saeedabadian A. Influences of lactose hydrolysis of milk and sugar reduction on some physical properties of ice cream. J Food Sci Technol 2015;52:367–74.
5. Vasiljevic T, Jelen P. Lactose hydrolysis in milk as affected by neutralizers used for the preparation of crude β-lgalactosidase extracts from Lactobacillus bulgaricus 11842. Innov Food Sci Emerg 2002;3:175–84.
6. Tari C, Ustok IF, Harsa S. Optimization of the associative growth of novel yoghurt cultures in the production of biomass, β-galactosidase and lactic acid using response surface methodology. Int Dairy J 2009;19:236–43.
7. Parashar A, Jin Y, Mason B, Chae M, Bressler DC. Incorporation of whey permeate, a dairy effluent, in ethanol fermentation to provide a zero waste solution for the dairy industry. J Dairy Sci 2016;99:1859–67.
8. Mahoney RR. Modification of lactose and lactose-containing dairy products with β-galactosidase. Developments in Dairy Chemistry-3. England: Elsevier, 1985:69–110.
9. Vasilevaa N, Ivanovb Y, Damyanovaa S, Kostovaa I, Godjevargovab T. Hydrolysis of whey lactose by immobilized β-galactosidase in a bioreactor with a spirally wound membrane. Int J Biol Macromol 2016;82:339–46.
10. Furlan SA, Schneider AL, Merkle R, Jonas MF, Jonas R. Formulation of a lactose-free, low-cost culture medium for the production of β-D-galactosidase by Kluyveromyces marxianus. Biotechnol Lett 2000;22:589–93.
11. Pivarnik LF, Senecal AG, Rand AG. Hydrolytic and transgalactosylic activities of commercial beta-galactosidase (lactase) in food processing. Adv Food Nutr Res 1995;38:1–102.
12. Chaverri P, Castlebury LA, Overton BE, Samuels GJ. Hypocrea/Trichoderma: species with conidiophore elongations and green conidia. Mycologia 2003;95:1100–40.
13. Fiederek J, Iliczuk Z. Screening of microorganisms for improvement of β-galactosidase production. Acta Microbiol Pol 1990;39:37–42.
14. Park YK, Santi MS, Pastore GM. Purification and characterization of β-galactosidase from Aspergillus oryzae. J Food Sci 1979;44:100–3.
15. Giacomini C, Villarino A, Fraqus LF, Batista-Vierra F. Immobilization of β-D-galactosidase from Kluyveromyces lactis on silica and agarose: comparison of different methods. J Mol Catal B: Enzym 1998;4:313–27.
16. Guimarães PM, Teixeira JA, Domingues L. Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnol Adv 2010;28:375–84.
17. Seyis I, Aksoz N. Production of lactase by Trichoderma sp. Food Technol Biotech 2004;42:121–4.
18. Jackson EH, Jelen P. Batch hydrolysis of lactose in concentrated whey systems. J Food Sci 1989;54:1086–7.
19. Szczodrak J. Hydrolysis of lactose in whey permeate by immobilized β-galactosidase from Penicillium notatum. Acta Biotechnol 1999:19:235–50.
20. Hatzinikolau DG, Katsifas E, Mammab D, Karagouni AD, Christakopoulos P, Kekos D. Modeling of the simultaneous hydrolysis–ultrafiltration of whey permeate by a thermostable β-galactosidase from Aspergillus niger. Biochem Eng J 2005;24:161–72.
21. Voorde V, Goiris K, Syren E, Van den Bussche C, Aerts G. Evaluation of the cold-active Pseudolosteromonas haloplanktis β-galactosidase enzyme for lactose hydrolysis in whey permeate as primary step of D-tagatose production. Process Biochem 2014;49:2134–40.
22. Oliveira C, Guimarães PM, Domingues L. Recombinant microbial systems for improved β-galactosidase production and biotechnological applications. Biotechnol Adv 2011;29: 600–9.
23. Tamriseven A, gan S. A novel method for the immobilization of β-galactosidase. Process Biochem 2002;38:27–30.
24. Mürschbächner AP, Volpato G, de Souza CF. Kluyveromyces lactis β-galactosidase immobilization in calcium alginate spheres and gelatin for hydrolysis of cheese whey lactose, Ciência Rural, Santa Maria 2016;46:921–6.
25. Szczodrak J. Hydrolysis of lactose in whey permeate by immobilized β-galactosidase from Kluyveromyces fragilis. J Mol Catal B Enzym 2000;10:631–7.