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Production of Lactase by *Trichoderma* sp.

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Summary

In order to find an alternative fungal source, 13 different fungi (*Aspergillus*, *Trichoderma*, *Penicillium*, *Rhizopus* and *Fusarium* sp.) were cultured in lactase production medium at 30 °C and 150 rpm for 6 days. Experimental results showed that *Trichoderma viride* ATCC 32098 has maximum lactase specific activity, followed by *Trichoderma harzianum* 1073 D3. In addition, the studies of stability were carried out in the pH range of 3.0–7.5 at the temperature between 20 and 70 °C. It was observed that the activity of lactase produced from *T. viride* ATCC 32098 was above 90 % in the pH range of 3.0–7.5 at the temperature between 20 and 60 °C, and even 66 % at 70 °C. It was concluded that *Trichoderma* sp., especially *T. viride* ATCC 32098, could be used as an alternative for the production of lactase in industrial scale.

Key words: lactase, *Trichoderma*, pH stability, temperature stability, fungus

Introduction

Milk, a vital nutrient for all living beings, contains lactose, proteins, fat, vitamins and minerals such as calcium and phosphorus. Among these, lactose, as the main carbohydrate in milk, is a type of sugar that is the major carbon source during growth (*1*). Lactase (EC 3.2.1.23) hydrolyses lactose into glucose and galactose (*2,3*). In case of shortage of lactase in small intestine, lactose cannot be hydrolysed, which is called lactose intolerance (*4,5*). For this reason, majority of the world population encounters problems due to their consumption of foods containing high amount of lactose. Therefore, pre-processing of milk and dairy products with lactase to hydrolyse lactose is necessary in order to eliminate this disadvantage (*6*).

On the other hand, although whey, which is a by-product in cheese industry, contains high amount of lactose, it cannot be utilised effectively. In order to make use of whey, lactose should be hydrolysed into glucose and galactose prior to any process. From the environmental point of view, it is better to use whey than dispose of it. As a result, lactase is essential for many processes in various industries, and therefore efficient and especially economical production of this enzyme was studied (*6,7*).

Lactase has attracted the attention of many researchers as it has a wide area of application in many industries. This enzyme can be produced from different sources such as bacteria, yeast and mould. Commercial lactases are produced from both yeasts, such as *Kluyveromyces lactis* and *Kluyveromyces fragilis*, and moulds, such as *Aspergillus niger* and *Aspergillus oryzae* (*1,2*). Despite the fact that many techniques have been developed based on different microorganisms, there is still a need for alternative enzyme sources. For example, in dairy industry lactases that are stable in a wide pH and temperature range should be used.

In this study, production of lactase from alternative fungal sources was investigated. First of all, lactase ac-

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tivities in different fungal sources were compared and the most efficient fungus for lactase production was determined. Then, pH and temperature stability of the produced enzyme was studied.

Materials and Methods

Microorganisms

In the study, *Trichoderma harzianum* and *Trichoderma viride*, which were isolated and classified from the microflora of Turkey by Marmara Research Center, were used for the production of lactase. These fungal strains were *T. harzianum* 1073 D3, *T. harzianum* 1567 D2, *T. harzianum* 1620 D2, *T. harzianum* 1041 D1, *T. viride* 1897 A2 and *T. viride* ATCC 32098. In addition, *A. niger*, *Rhizopus*, *Penicillium* sp., *P. spinulosum*, *T. viride*, *Fusarium culmorum* and *F. accumulatum* strains from Hacettepe University Microbiology Laboratory were used. Stock cultures were produced on potato dextrose agar in 4–5 days and maintained at 4 °C.

Medium

Medium described by Fiedurek and Ilczuk (*8*) was used with some modifications for growth and enzyme production. Medium contains (as g/L): lactose 10.0, peptone 1.5, yeast extract 1.0, KH_2PO_4 1.0, $(NH_4)H_2PO_4$ 7.0, $MgSO_4 \cdot 7H_2O$ 1.0 and $CaCl_2$ 0.3. In 250-mL flasks, 50 mL of media, pH=5, were sterilised in autoclave at 121 °C and 1.52 bar for 15 min.

Inoculation

Spore suspensions of 1 mL containing $15 \cdot 10^6$ spores were inoculated into 50-mL growth medium at pH=5.0 and incubated at 30 °C and 150 rpm for 6 days.

Determination of growth

The amount of growth in cultures was calculated as dry weight by filtration method (*9*).

Determination of enzyme activity

Lactase activity was determined by the method described by Reczey *et al.* (*10*). The culture filtrate was centrifuged at 7 200 rpm for 15 min and used as the enzyme sample. The enzyme was assayed with 2.5 mg/mL of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate, which was prepared in 0.1 M sodium-acetate buffer, pH=5. Then 1 mL of substrate solution was incubated with 0.2 mL of sample at 50 °C for 5 min. Reaction was terminated by adding 1 mL of 10 % sodium carbonate into the reaction tube. The absorbance was read at 420 nm using Jenway, model 6105 UV-Vis spectrophotometer. The amount of *o*-nitrophenol was calculated from the standard curve. In addition, when lactose was used as substrate for determining lactase activity in *T. viride* ATCC 32098, the liberated glucose was estimated with Trinder Reagent (Sigma). One unit of lactase activity was described as the amount of enzyme producing 1 μmol of glucose or *o*-nitrophenol in 1-mL medium at 50 °C in 1 min. For specific activity calculations, the amount of protein was determined by Lowry method (*11*).

Stability of lactase

pH stability

Stability of the enzyme at various pH values was studied by incubating the enzyme in the respective buffers (pH=3.0–7.5) for 1 h at room temperature prior to the addition of substrate. The enzyme was then assayed for lactase activity under standard assay conditions at 50 °C and pH=5.0. Enzyme activities were determined as relative activities.

Temperature stability

In order to determine the temperature stability, lactase in the absence of substrate was kept at temperatures between 20 and 70 °C for 1 h. After adding the substrate, activity was determined by the standard method (50 °C, pH=5.0). Enzyme activities were determined as relative activities.

Results and Discussion

In order to find an alternative fungal source, microorganisms that produce extracellular enzymes efficiently were determined. In this respect, 13 different fungi (*A. niger*, *T. harzianum* 1567 D2, *T. harzianum* 1073 D3, *T. harzianum* 1620 D2, *T. harzianum* 1041 D1, *T. viride* 1897 A2, *T. viride* ATCC 32098, *T. viride*, *Penicillium* sp., *P. spinulosum*, *Rhizopus* sp., *Fusarium culmorum* and *F. accumulatum*) were cultured in lactase production medium at 30 °C and 150 rpm for 6 days. After the incubation, lactase activities, growth and the amount of protein were determined (Figs. 1 and 2).

In similar studies found in literature, lactase production from *A. niger*, *Penicillium* and *Fusarium* sp. were investigated (*6,8,12*). High enzyme activity was observed in studies carried out with *P. notatum* and *P. chrysogenum* (*3,8*) but no studies were found on *P. spinulosum*, which

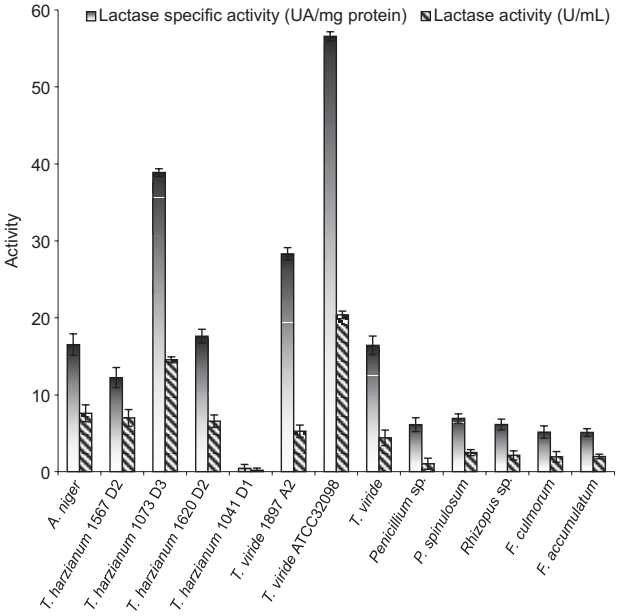

Fig. 1. Lactase activities of different fungi. Results are average values of three parallel studies. Standard deviation is shown on the graph

we used. Similarly, when studies with *Fusarium* sp. were investigated, it was concluded that *F. moniliforme* was a potent lactase producer *(12)* but no promising results on *F. accumulatum* and *F. culmorum*, which we studied, were found. In this respect, among the above mentioned studies, lactase activities were found to be varying between 1.94–16.23 U/mL (*2*,*3*,*8*,*13*).

Our experimental results showed that *T. viride* ATCC 32098 has maximum lactase activity and lactase specific activity, followed by *T. harzianum* 1073 D3 (Fig. 1). In previous studies *Aspergillus*, *Penicillium* and *Fusarium* were used (*8*,*12*,*14–16*). However, our results revealed that activities of these fungi were relatively low despite their high growth rate. In literature, although *Trichoderma* sp. was found to be effective source for different enzymes (*17–19*), no studies on lactase production from *T. harzianum* and *T. viride* could be found. In our study, lactase specific activities of *Trichoderma* sp. were determined to be comparatively high, except of *T. harzianum* 1041 D1, which is probably due to the fact that lactase production of this fungus can be intracellular. As a result, *T. viride* ATCC 32098 was selected as the most suitable fungus for the rest of the study. The enzyme produced by this microorganism is extracellular, therefore it is economically preferable especially in commercial applications, as it eliminates the costs induced by extracting the enzyme from the cell. *Trichoderma* sp. can produce among others xylanase, cellulase, and lactase. Therefore, in order to make use of lignocellulosic agricultural wastes, the production of lactase from *Trichoderma* sp. is advantageous.

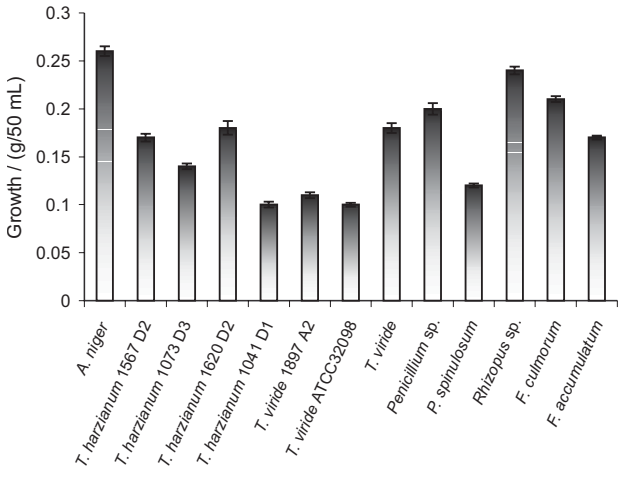

Fig. 2. Growth of different fungi. Results are average values of three parallel studies. Standard deviation is shown on the graph

Although the growth of *A. niger* and *Rhizopus* sp. is high when compared to other fungi, as it can be seen from Fig. 2, their activities are low. The reason for this may be that these fungi use peptone and yeast as carbon sources as well as lactose, while growth rates are high when compared to other fungal sources. The reason for low production of lactase may be because the enzyme is intracellular.

As stated above, *Trichoderma viride* ATCC 32098 has maximum lactase specific activity and lactase activity, therefore its lactase production was investigated in the rest of the study. In this respect, enzyme activity was also determined using lactose as substrate and found to be 29.54 U/mL. In other words, as lactase is mainly used in milk and whey, the activity was also investigated using lactose as substrate.

On the other hand, stability is an important criterion for an enzyme intended for use in industrial scale. Therefore, in the second part of the study, pH and temperature stability of lactase were investigated. For an enzyme, pH stability is required both for usage and storage purposes. For example, due to their varying activities in different pH values, enzymes produced from moulds are used in whey and the ones produced from yeasts are used in milk. However, our experimental results showed that enzyme activity was above 90 % in the pH range of 3.0–7.5 (Fig. 3), which implies that the produced enzyme can be used both in whey, having acidic pH, and in milk, having neutral pH. Parallel to our findings, in a previous study with *Rhizomucor* enzyme activity was above 90 % in the pH range 3.5–7.5 (*16*). In another study carried out with *T. lanuginosus*, enzyme was stable in the pH range 6.0–9.0 (*20*).

Enzymes that are stable at high temperatures should be preferred in processes where microbial contamination should be eliminated and high reaction rate is desired, therefore lactases used especially for industrial purposes need to be stable at high temperatures. When temperature stability was investigated, it can be seen in Fig. 4

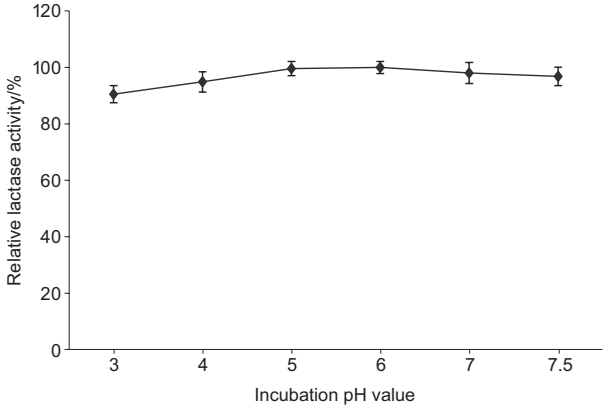

Fig. 3. pH stability of lactase. Results are average values of three parallel studies. Standard deviation is shown on the graph

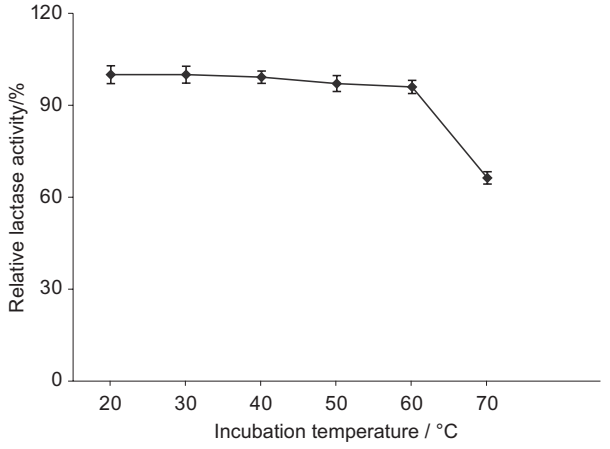

Fig. 4. Temperature stability of lactase. Results are average values of three parallel studies. Standard deviation is shown on the graph

that the enzyme activity was above 95 % at the temperatures between 20–60 °C and 66 % at 70 °C after 1 h. These results imply that the produced lactase can be used for different purposes in a wide temperature range. In a previous study it was reported that enzymes produced from moulds were more stable from the temperature point of view when compared to the ones produced from yeasts (*8*). On the other hand, it was reported in several studies that lactase produced from yeasts lost its activity at above 40 °C (*12,21*).

Conclusion

It can be concluded that *Trichoderma* spp. are effective alternatives for the production of lactase. In this study, *T. viride* ATCC 32098 was found to be the most potent lactase producer, followed by *T. harzianum* 1073 D3. In addition, both temperature and pH stability of the produced lactase were considerably high at temperatures between 20 and 60 °C and pH=3.0–7.5 range, which implies that it can be used in industrial scale for various applications, especially in dairy industry.

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Proizvodnja laktaze iz *Trichoderma* sp.

Sažetak

Da bi se našao alternativni fungalni izvor laktaze, uzgojeno je 13 različitih fungi (*Aspergillus*, *Trichoderma*, *Penicillium*, *Rhizopus* i *Fusarium* sp.) u podlozi za proizvodnju laktaze pri 30 °C tijekom 6 dana i 150 o/min. Dobiveni rezultati pokazuju da *Trichoderma viride* ATTC 32098 ima maksimalnu specifičnu aktivnost laktaze, a slijedi ju *Trichoderma harzianum* 1073 D3. Osim toga provedena su ispitivanja stabilnosti enzima pri pH od 3,0 do 7,5 i temperaturnom području od 20 do 70 °C. Aktivnost laktaze, proizvedene u *T. viride* ATTC 32098, bila je iznad 90 % pri pH=3,0–7,5 i 20–60 °C, a pri 70 °C iznosila je 66 %. Zaključeno je da *Trichoderma* sp., a posebno *T. viride* ATTC 32098, može poslužiti za proizvodnju laktaze u industrijskom mjerilu.