PAPER

CHARACTERIZATION OF BETA-GALACTOSIDASE FROM *ALTERNARIA ALTERNATA* ON SOLID-STATE CULTIVATION

CARATTERIZZAZIONE DI BETA-GALATTOSIDASI DA *ALTERNARIA ALTERNATA* SU TERRENO DI COLTURA SOLIDO

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ABSTRACT

The effects of orange peel, lemon peel, apple peel, wheat-bran and watermelon peel on beta-galactosidase production from *Alternaria alternata* were studied. Beta-galactosidase (EC-3.2.1.23) was produced best by solid-state fermentation of *A. alternata* on wheat bran. The effects of pH and incubation temperature were studied. The highest enzyme activity was obtained at 50°C and pH 5.0. The enzyme was relatively stable in the pH range 3.0-5.0 and between 20°-50°C. The enzyme maintained 85%

RIASSUNTO

Sono stati studiati gli effetti della buccia di arancia, di limone, di mela, di cocomero e della crusca di grano sulla produzione di beta-galattosidasi da *Alternaria alternata*. La beta-galattosidasi (EC-3.2.1.23) veniva meglio sintetizzata dall'*A. alternata* per fermentazione su stato solido sulla crusca di grano. Sono stati studiati gli effetti del pH e della temperatura. L'attività enzimatica più elevata è stata osservata a 50°C e a pH 5,0. L'enzima era relativamente stabile a pH compresi fra 3,0-5,0 e ad

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of its activity at 20°-30°C and 70% at 50°C. Activity was more than 92% between pH 3.0-5.0 and 42% in 40 min and 50% in 30 min at 60°C. In addition, glucose did not have an inhibitory effect, and galactose had only a slight inhibitory effect.

temperature comprese fra 20° e 50°C. L'enzima manteneva l'85% della sua attività a 30°-50°C e il 70% a 50°C. L'attività era maggiore del 92% a valori di pH compresi fra 3,0-5,0 e risultava del 42% dopo 40 min e del 50% dopo 30 min a 60°C. Inoltre, il glucosio non presentava alcun effetto inibitorio, mentre il galattosio presentava solo un leggero effetto inibitorio.

INTRODUCTION

Many microorganisms, including bacteria, yeast and fungi produce different groups of enzymes. Enzymes are among the most important products obtained through microbial sources and many industrial processes use enzymes at some stage of the process (PANDEY *et al.*, 1999). Among these enzymes, beta-galactosidase (EC 3.2.1.23), also called lactase, catalyzes the hydrolysis of β-1,4-D-galactosidic linkages such as those in lactose. This enzyme can be found in plants, animals and microorganisms (PISANI *et al.*, 1990; FIEDUREK and ILCZUK, 1990; GUVEN *et al.*, 2007).

Lactose is the main component in milk and whey and the consumption of foods with a high content of lactose causes problems for lactose-intolerant people (GUVEN *et al.*, 2007; RICHMOND *et al.*, 1981). To avoid this problem, lactose needs to be hydrolyzed into simpler sugars such as glucose and galactose. The increasing demand for lactose-free dairy products is expected to create a world demand for beta-galactosidase (PETRIDES *et al.*, 1999). Beta-galactosidase, with the help of lactose hydrolysis, can also resolve the problem of pollution caused by whey disposal (CHOLLANGI and HOSSAIN, 2007).

The economics of enzyme-producing processes is one of the major factors that should be taken into account in a competitive market. The economical production of these enzymes has received increasing attention by many researchers. Solid-state fermentation has tremendous potential for producing enzymes economically; it is of special interest in processes where the crude fermented product can be used directly as the enzyme source (PANDEY *et al.*, 1999).

The aim of this study was to determine the optimum conditions that affect the beta-galactosidase enzyme activity produced by *Alternaria alternata* on solid-state cultivation medium; the stability of the enzyme was also investigated.

MATERIALS AND METHODS

Microbial strain

A. alternata strain from the Microbiology Laboratory of Hacettepe University was used to produce beta-galactosidase enzyme. Stock cultures were maintained on potato dextrose agar at +4°C.

Medium and inoculation

The medium described by FIEDUREK and ILCZUK (1990) was used with some modifications to produce and grow the enzyme. The medium contains (as g/L); 10.0 lactose, 1.5 peptone, 1.0 yeast-extract, 1.0 KH_2PO_4, 7.0 $(NH_4)H_2PO_4$, 1.0 $MgSO_47H_2O$ and 0.3 $CaCl_2$. Fifty mL of

media at pH 5.0 were placed in 250 mL flasks and sterilized in an autoclave at 121°C, 1.5 atm for 15 min. Wheat bran was used for solid-state cultivation. The enzyme was produced by solid cultivation using a 250 mL Erlenmeyer flask containing 10 g wheat bran and 10 mL distilled water. Solid medium was sterilized in an autoclave at 121°C, 1.5 atm for one h. The crude-extract was obtained after centrifugation. Wheat-bran, which is the substrate used in the solid-state media, was replaced by dehydrated lemon, orange, watermelon, apple and watermelon peel. The effect of lemon, orange, watermelon, apple, watermelon peel and wheat bran on beta-galactosidase production was studied. Spore suspensions containing $3 \bullet 10^6$ spores/mL were inoculated onto the media and incubated at 30°C for 6 d.

Enzyme activity assays

Beta-galactosidase activity was assayed by the method described by RECZEY *et al.* (1992). The culture was centrifuged at 7,200 rpm for 15 min and supernatant was used as the enzyme sample. The enzyme was assayed with 2.5 mg/mL o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate (Merck, Darmstadt), which was prepared in 0.1 M sodium-acetate buffer (pH 5.0). One mL of substrate solution and 0.2 mL enzyme sample were then added to the reaction tubes. The reaction mixture was incubated at 50°C for 15 min. Reaction was terminated by adding one mL of 10% sodium carbonate. The absorbance values were determined at 420 nm using a Jenway (Essex, UK), 6105 u.v., vis spectrophotometer. The amount of o-nitrophenol was calculated from the standard curve plotted in the 10-90 μg range. In addition, when lactose was used as substrate to determine lactase activity, the liberated glucose was estimated with Trinder Reagent (Sigma, USA).

One unit of beta-galactosidase activity was defined as the amount of enzyme that releases one μmole of o-nitrophenol or glucose per min in one mL medium at 50°C under standard assay conditions.

Characterization of beta-galactosidase enzyme

The medium was incubated 6 days at 30°C and the supernatant of *A. alternata* was used as the enzyme source. To investigate the effect of temperature on enzyme activity, the reaction medium was incubated at different temperatures between 20°-60°C. Similarly, in order to determine the optimum pH value, incubations were carried out at different pH values between 4.0-6.0. Enzyme activities were determined as relative activity.

Temperature stability

Enzyme stabilities at different temperatures (between 20°-60°C) were determined after preincubation for one hour, cooling and carrying out the standard assay procedure. In order to determine the stability of the enzyme at 60°C, enzyme activities were measured at 20, 30 and 40 min.

pH stability

Enzyme stabilities in the pH range 3.0-7.0 were determined with the standard assay procedure after preincubation for one hour. In this way, enzyme activities were determined as relative activity.

Effect of glucose and galactose on beta-galactosidase activity

One hundred mM glucose and galactose were prepared separately in 0.1 M sodium acetate buffer (pH 5.0). One mL of each solution was added to the reaction tubes containing 0.5 mL enzyme and 1 mL ONPG solution. The percent activity was determined based on the reference sample, which did not contain glucose or galactose. In this way, enzyme activities were determined as relative activity.

RESULTS AND CONCLUSIONS

Beta-galactosidase production from *A. alternata* in solid-state culture was determined with the different substrates. There are numerous reports in the literature on producing beta-galactosidase enzyme from different fungi such as *Aspergillus, Penicillium*, *Trichoderma* and *Alternaria* sp. In these studies, whey and liquid media were used for enzyme production (MACRIS, 1982; BAILEY and LINKO, 1990; FIEDUREK *et al.*, 1996; NAGY *et al.*, 2001; SEYIS and AKSOZ, 2004). In contrast, solid-state fermentation requires only simple fermentation equipment and offers numerous advantages including high productivity at relatively higher concentrations (PANDEY *et al.*, 1999).

Enzyme production in solid state and liquid cultivation was compared and the results show that solid-state gives higher yields (solid state: 6.99 U/mL; liquid: 4.90 U/mL). In a previous study carried out with *Rhizomucor*, the rate of enzyme production in solid-state medium was nine times faster compared with submerged fermentation (SHAIKH *et al.*, 1997). Therefore, the major objective of this study was to economically produce an industrial enzyme on solid-state production media. A similar approach has been followed in other studies on the same subject (PARK *et al.*, 1979; PASTORE and PARK, 1979; BARKER and SHIRLEY, 1980; BAILEY and LINKO, 1990; GONZALES and MONSAN, 1991; SOUZA *et al.*, 2001; HATZINIKOLAOU *et al.*, 2005; SZENDEFY *et al.*, 2006; SHANKAR and MULIMANI, 2007; XIONG *et al.*, 2007).

In liquid fermentation, lactose is used as an inducer and lactase is produced in the media. Lactase enzyme produced by solid-state fermentation from *A. alternata* shows that this enzyme is not induced by lactose but can be produced constitutively even if there is no lactose in the media.

The enzyme was then produced in different solid-state cultivation media and the results were compared. Agro-industrial residues are usually considered the best substrates for solid-state fermentation. The results show that medium containing wheat bran had the highest enzyme yield. The relatively low activities in the other media may have been due to the fact that *A. alternata* may synthesize more suitable enzymes in these media (Fig. 1). In a study to produce beta galactosidase from *Aspergillus oryzae* with submerged cultivation, the highest level of activity was obtained on the wheat bran medium (BAILEY and LINKO, 1990). To date, no studies have reported the use of substrates other than wheat bran. Therefore, the production of a considerable amount of beta galactosidase on lemon peel is a promising result.

Another objective of the study was to characterize the enzyme. To do this, the physiological conditions of the reaction medium were investigated. In lactase activity studies, chromogenic substrate ONPG was used in the reaction medium rather than lactose in order to color the product (DESHPANDE *et al.*, 1989; PISANI *et al.*, 1990). In the present study, ONPG was used as substrate for enzyme characterization. This enzyme is used in milk and dairy products and therefore lactose is the natural substrate of this enzyme. The Km value for lactose was calculated and found to be 6.87 mM.

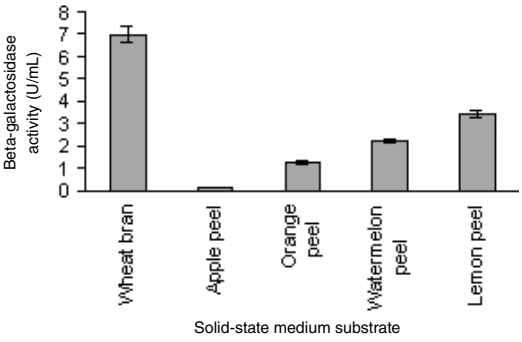

Fig. 1 - Beta-galactosidase production on alternative solid-state media.

When incubation temperatures between 20°-60°C were used, it was observed that the maximum activity was reached at 50°C, which can be considered the optimum temperature (Fig. 2). In previous studies, the optimum temperature for maximum beta-galactosidase activity was reported to be 50°C (RIOU *et al.*, 1992; SEYIS and AKSOZ, 2004; NAKKHARAT and HALTRICH, 2006; PANESAR *et al.*, 2006). Enzymes that can operate at higher temperatures have numerous advantages in biotechnological applications (YANO and POULOS, 2003). Therefore, a maximum activity at 50°C is an advantage of this enzyme, especially when used in commercial applications.

The effect of pH on beta-galactosidase activity was also determined at pH values between 4.0 and 6.0. The results of the experiments showed that the optimum pH was around 5.0 (Fig. 3). In similar studies carried out with fungal sources, the optimum pH values were between 4.0-5.0 (PARK *et al.*, 1979; FIEDUREK and ILCZUK, 1990; ISMAIL *et al.*, 1992; RIOU *et al.*, 1992; SEYIS and AKSOZ, 2004).

In a number of previous studies, the characteristics of enzymes produced in liquid culture were compared with those produced in solid-state cultures. The biochemical characteristics of the enzymes differed, which indicates that the culture method has a considerable effect on the biochemical characteristics of the enzyme (DESCHAMPS and HUET, 1985; ACUÑA-ARGÜELLES *et al.*, 1995; SARA SOLÍS-PEREIRA *et al.*, 1993; VINIEGRA-GONZALEZ *et al.*, 2003). In this study, temperature and pH characteristics of the enzyme produced in solid-state culture were compared with the results of a previous study (SEYIS, 2003), in which the enzyme was produced in liquid media; the temperature and pH optima for the enzyme were almost the same in both media.

The industrial use of an enzyme is often limited due to lack of stability. For enzymes used in commercial applications, the temperature and pH ranges at which the enzyme is relatively stable are important for the efficiency of the process in which they are used.

The temperature stability of the beta-galactosidase enzyme produced was studied and the results show that the enzyme kept more than 70% of its original activity between 20°-50°C (Fig. 4). When temperature stabilities were examined in liquid media, it was observed that the enzyme activity was approximately 50% at 50°C (SEYIS, 2003).

In a similar study, beta-galactosidase was produced from *P. notatum*. The results show that the stability of the enzyme was high between 20°-40°C and decreased above 50°C after 1 h of

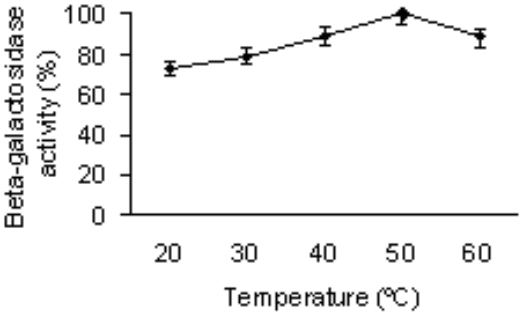

Fig. 2 - Effect of temperature on beta-galactosidase activity.

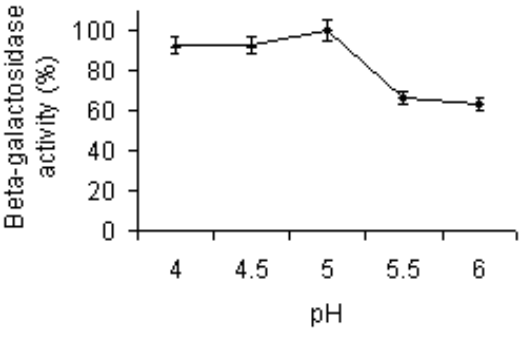

Fig. 3 - Effect of pH on beta-galactosidase activity.

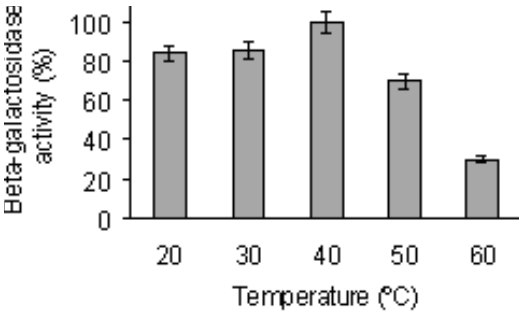

Fig. 4 - Stability of beta-galactosidase at 20°-60°C.

incubation (FIEDUREK and ILCZUK, 1990). It was reported that the activity of beta-galactosidase produced on *Aureobasidium pullulans* decreased rapidly above 45°C (DESHPANDE *et al.*, 1989). In another study carried out with *B. bassiana*, the activity decreased to 5.9% after 30 min at 60°C (MACPHERSON and KHACHATOURIANS, 1991). Results of the stability studies on *Aspergillus* and *Penicillium* at different incubation temperatures show that the stabilities of the enzymes change considerably after one h of pre-incubation (ROGALSKI *et al.*, 1994).

It was observed that at 60°C the enzyme maintained 50% of its original activity after 30 min and 42% after 40 min (Fig. 5). This is due to the fact that the structure of the enzyme changes at high temperatures. On the other hand, it was reported that the activities of enzymes produced from yeasts decrease markedly above 40°C when compared with the activities of the enzymes produced from fungi (SORENSEN and CRISAN, 1974). Therefore, beta-galactosidase enzyme production on yeasts is not suitable if they are to be used at high temperatures. When the enzyme activity at 60°C in liquid culture (SEYIS, 2003) was investigated, it was observed that after 40 min the activity was 53% of the original activity.

When pH stability of the enzyme was investigated, it was observed that the activity decreased at pH 6.0 and 7.0, while at pH 3.0 and 4.0 the enzyme activity was greater than 92% after one h. These results imply that this enzyme is highly stable with respect to pH (Fig. 6). In contrast, the stability of the enzymes changed considerably in studies carried out with *Aspergillus* and *Penicillium* at different pH values (ROGALSKI *et al.*, 1994).

Finally, the effect of glucose and galactose on beta-galactosidase activity was investigated. The results (Fig. 7) show that galactose has an insignificant inhibition effect and glucose does not have any. Similarly, in a previous study, in which the enzyme was produced on *Trichoderma viride*, the activity increased slightly with glucose and was not inhibited significantly with ga-

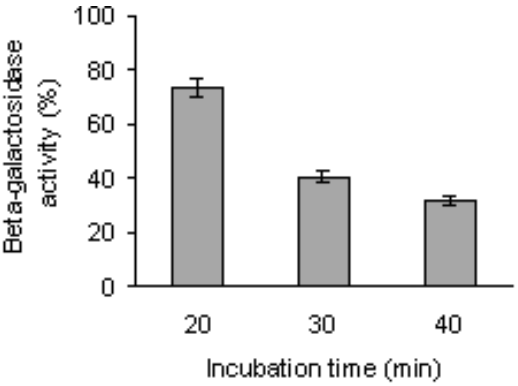

Fig. 5 - Effect of incubation time on beta-galactosidase activity at 60°C.

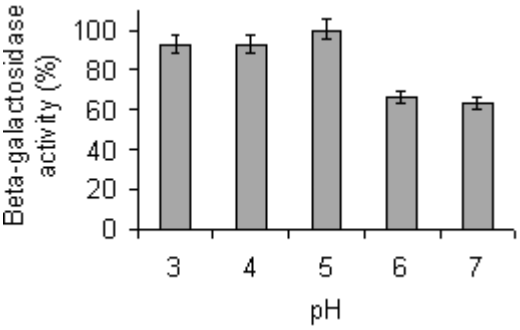

Fig. 6 - Stability of beta-galactosidase at pH 3-7.

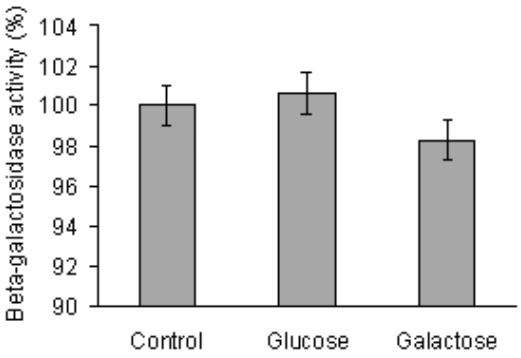

Fig. 7 - Effect of glucose and galactose on beta-galactosidase activity.

lactose (SEYIS, 2004). The literature reports that glucose and galactose act as inhibitors of beta-galactosidase enzyme produced from *Kluveromyces lactis* (CAVAILLE and COMBES, 1995). In studies carried out with *Bacillus* sp., the enzyme was strongly inhibited by galactose (CHAKRABORTI *et al.*, 2000; CHAKRABORTI *et al.*, 2003).

When the results from a solid-state medium are compared with those from a liquid medium (SEYIS, 2003), it can be concluded that solid-state cultivation is preferable. For example, when temperature stability is considered between 20°-50°C, enzyme activity was greater than 70% in solid state but approximately 50% in liquid medium. The enzymes were more stable at high temperatures and over a wide temperature range. This is very advantageous from an economical point of view for industrial applications. However, when pH stabilities were compared, the enzymes produced in liquid medium were more stable, which can be considered as a minor disadvantage of solid-state cultivation.

The optimum temperature and pH for beta-galactosidase activity were 50°C and 5.0, respectively. It was observed that, the enzyme was stable in the pH range 3.0-7.0 and between 20°-50°C. At 60°C the activity decreased to 42% after 30 min and to about 33% after 40 min.

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