Research Article

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Cultural conditions optimization for production of β-galactosidase from Bacillus licheniformis ATCC 12759 under solid-state fermentation

Katı Faz Fermantasyonu altında Bacillus licheniformis ATCC 12759’dan β-Galaktosidadın Üretimi için Kültürel Şartların Optimizasyonu

Abstract

Objective: The aim of this work was to study the optimal cultivation conditions for β-galactosidase production by Bacillus licheniformis ATCC 12759.

Materials and methods: The screening of β-galactosidase production from B. licheniformis ATCC 12759 was performed by solid state fermentation method on media rich with rice bran (RB). Different factors were tested for the optimization of β-galactosidase production.

Results: Certain fermentation parameters involving incubation time, incubation temperature, inoculum level, moisture content, initial pH, agitation speed, size of fermentation medium and optimum temperature of β-galactosidase activity were studied separately. Maximal amount of β-galactosidase production was obtained when solid-state fermentation (SSF) was carried out using RB, having inoculum level 35%, moisture content of 20%, initial pH 7.5 at 37°C for 48 h.

Conclusion: Results indicated that optimal fermentation conditions play a key role in the maximum production of β-galactosidase from B. licheniformis ATCC 12759. This study shows the potential of the studied enzymes to be promoting candidates for the degradation of lactose and production of important bioproducts.

Keywords: Bacillus licheniformis; β-galactosidase; Fermentation; Optimization; Rice bran.

Introduction

β-galactosidase [EC3.2.1.23] catalyzes the hydrolytic process of β-1,4-D-galactosidic linkages found in lactose (β-D-galactopyranosyl-(1→4)-D-glucopyranose) and releases D-glucose and D-galactose as an end product [1]. As a main milk sugar, lactose needs to be sufficiently metabolized to fulfill the energy demand of human beings and makes possible the consumption of milk and other dairy products.

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other dairy products by lactose intolerant people [2]. In addition, some β-galactosidases can transfer the galactosyl residues of lactose to saccharide acceptors to yield galactooligosaccharides (GOS) [3–5]. GOS is an important prebiotic which can improve gut health by stimulating the proliferation probiotic intestinal bacteria [5]. Some recent studies have demonstrated that GOS can positively modify the immune function of overweight adults and increase calcium absorption of young girls [6–8].

The sources of β-galactosidase are extensively distributed in nature, namely in microorganisms, plants and animal organs [9, 10]. β-galactosidases from microbial sources exhibit a great industrial relevance mainly due to their easy handling, greater catalytic activity and high production yield [11]. As a result of commercial interest in β-galactosidase, a large number of microorganisms have been assessed as potential sources of this enzyme.

Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH [12]. But, nowadays, the demand for β-galactosidase production, as well as the development of an effective and inexpensive β-galactosidase production process has increased significantly. Industrial production of enzymes can be made economical by utilizing low-cost substrates such as agricultural byproducts in the production medium [13]. Compared with SmF, solid-state fermentation (SSF) has many advantages, such as superior productivity, greater simplicity, lower capital investment, less energy requirement and waste water output, better product recovery, etc., and is reported to be the most appropriate process for developing countries [14]. On preliminary cost analysis, a net savings of about 60 and 50% on fermentation medium cost and the expenditure on down-stream processing, respectively, as compared to the presently employed SmF technique was evident [15, 16]. It can be of special interest in those processes where the crude fermented products may be used directly as the enzyme sources for biocatalysis and biotransformation [17].

A reduction in β-galactosidase production cost is very important. The use of abundantly available lignocellulosic crop residues, such as rice husk, rice bran (RB), and wheat bran, for culture of microbes producing β-galactosidase offers an approach to reach this goal. In general, β-galactosidase is intracellular enzyme and to study intracellular enzymes is difficult. But β-galactosidase production of such microorganisms is very easy and economic because it is extracellular enzyme. This paper describes the screening of various agro-industrial substrates and the development of a suitable low cost fermentation medium for the optimizing following parameters with emphasis on incubation time, extraction medium, temperature, initial pH, moisture level, inoculum size, supplementation of carbon, nitrogen sources and metal salts by using RB as solid substrate.

### Materials and methods

#### Bacterial strains and culture conditions

β-Galactosidase producing *Bacillus licheniformis* ATCC 12759 which was procured from MicroBioLogics, Inc. was used as biological material. *Bacillus licheniformis* ATCC 12759 was grown on nutrient agar at 37°C for 24 h for inoculum preparation. A loopful of the growth was transferred to Laura broth (LB) liquid medium [1% yeast extract, 0.5% peptone, 0.5% NaCl, (w/v), pH 7.0].

#### Enzyme production in SSF

**Solid-state fermentation**

SSF was carried out by taking 3 g of dry substrate in a 100 mL Erlenmeyer flask to which distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121°C for 15 min. Flasks with inoculated were shaken at 150 rpm at 37°C for 144 h. The contents of the flasks were harvested and assayed every 24 h.

**Enzyme extraction**

The enzyme from the fermented bacterial bran was extracted twice with tap water. The slurry was squeezed through damp cheesecloth. Extracts were pooled and centrifuged at 4°C for 15 min at 10,000 rpm to separate small particles of different substrates, cells and spores. The brown, clear supernatant was used in enzyme assays.

**Enzyme assay**

The reaction mixture containing 500 μL 6 mM 2-nitrophenyl β-D-galactopyranoside in 0.1 M sodium phosphate buffer (pH 6.8) and 200 μL of enzyme solution was incubated for 30 min at 37°C. The reaction was ended by adding 0.5 mL of 1 M Na₂CO₃ and the concentration of
o-nitrophenol (ONP) released from ONPG was determined by measuring the absorbance at 420 nm, using a standard calibration curve. The enzyme activity was expressed as specific activity (U mg⁻¹ soluble protein) and one unit of β-galactosidase activity (U) was defined as the amount of enzyme that liberates 1 nmol ONP per minute [18]. All experiments were conducted in triplicate and the mean at three with standard deviation (SD) was represented.

**Effect of process parameters on β-galactosidase production in SSF**

The optimization of medium components and fermentation process is of primary importance in any fermentation process. Combinations of the best substrates were employed for further optimization of process parameters, namely initial moisture content (20, 30, 40, 50 and 60%), incubation time (24, 48, 72, 96, 120 and 144 h), incubation temperature (30, 37, 40, 45 and 50°C), initial pH of the medium (pH: 4.0–10.0), inoculum size (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6 and 8 × 10⁶ CFU/mL), agitation speed (60, 100, 120, 150, 180 and 200 rpm), scale up (100, 250, 500 and 1000 mL shake flasks), the effects of the different proportions in mixed substrates, while nutrient supplementation such as inorganic nitrogen sources 1% (by mass) (ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulfate), organic nitrogen sources (peptone, tryptone, yeast extract, beef extract, urea, and casein), and added metal salts 0.1% (by mass) FeSO₄·7H₂O, MgSO₄·7H₂O, CuSO₄·5H₂O, ZnSO₄·7H₂O, and CaCl₂ were optimized. To study the efficacy of various inducers, the medium was supplemented independently with 1% mannose, xylose, lactose, sucrose, fructose, galactose, glucose, and arabinose. Distilled water, tap water, 50 mM NaCl, 0.1 M phosphate buffer (pH = 7.0), 1% solution of various detergents like Tween 40, CHAPS (3-(3-cholamidopropyl)-dimethylammonio)-propane-sulfonate), sodium dodecyl sulfate (SDS) and Triton X-100 were used independently to find the best extraction medium for the enzyme. Data were expressed as the average of three replicates.

**Results**

The time courses of β-galactosidase production by *B. licheniformis* ATCC 12759 are shown in Figure 1. The initial β-galactosidase biosynthesis by *B. licheniformis* ATCC 12759 was reached the maximum (3244.7 U/mg) at 120 h.

Investigation of the effect of temperature on enzyme production during fermentation showed that the optimum temperature for maximum yield of β-galactosidase (3396.8 U/mg) was 37°C (Figure 2).

From the results (Table 1), it is clear that among all the solvents, tap water (3239.0 U/mg) gave the best extraction of β-galactosidase from the fermented solids.

The highest enzyme production (3530.4 U/mg) was obtained at an inoculum level of 35% (v/w) (Figure 3). The

| Extraction medium   | Specific activity (U/mg) |
|---------------------|--------------------------|
| NaCl                | 2563.6 ± 35.1            |
| CHAPS               | 2539.9 ± 24.1            |
| Triton X-100        | 1773.5 ± 16.9            |
| SDS                 | 2505.3 ± 9.4             |
| Distilled water     | 2675.3 ± 110             |
| Phosphate buffer    | 2746.7 ± 18.4            |
| Tween 40            | 1742.7 ± 16.8            |
| Tap water           | 3229 ± 12.7              |

(p < 0.05).
results from this study indicate that 35% inoculum size was optimal, balancing enzyme and biomass production.

In the present investigation, five moisture levels ranging from 20 to 60% were established to study their effect on β-galactosidase production and the results obtained are shown in Figure 4. The highest production of β-galactosidase (3238.4 U/mg) was obtained when the initial moisture content was 20%.

Results showed that maximum enzyme production was observed at pH 7.0 (Figure 5). Therefore, in the subsequent experiments, the initial pH of the fermentation medium was adjusted to 7.0 with tap water.

β-galactosidase production was investigated at six different speeds (60–200 rpm). The optimal agitation speed for maximum β-galactosidase production (3473.3 U/mg) was obtained at 150 rpm (Table 2). The higher agitation levels (180–200 rpm) reduced β-galactosidase production from *B. licheniformis* ATCC 12759 due to sheer stress and heterogeneous mixing effects.

To improve for a large-scale SSF, β-galactosidase production was investigated in four different fermentation medium sizes (100, 250, 500 and 1000 mL Erlenmeyer flasks). When solid state fermentation with *B. licheniformis* ATCC 12759 was carried out in Erlenmeyer flasks of various sizes with corresponding increase in with different quantities of RB moistened with appropriate amounts of tap water, the time course of enzyme production was similar to that in 100 mL Erlenmeyer flasks containing 2 g of RB (control), in most of the cases. The results were quite encouraging for the large scale production of the enzyme though the yields exhibited slight decline with the increase in substrate quantity which is probably due to lesser degree of aeration (Table 3). Future research will be focused on optimizing the nutrient conditions to obtain higher biomass, which is desirable for the industrial development of β-galactosidase product under low production cost [19].

To examine the effect of carbon source on the production of β-galactosidase *B. licheniformis* ATCC 12759, which produced a high amount of β-galactosidase with the highest specific activity (Table 4). The production of β-galactosidase by *B. licheniformis* ATCC 12759 was suppressed when the bacterium was grown on readily metabolizable sugars, since a low basal activity of β-galactosidase was detected in the culture medium.

The production of β-galactosidase by *B. licheniformis* ATCC 12759 was suppressed when the bacterium was grown on different organic and inorganic nitrogen sources

![Figure 3: Effect of inoculum on β-galactosidase production by *B. licheniformis* ATCC 12759 in solid-state fermentation.](image)

![Figure 4: Effect of moisture level on β-galactosidase production by *B. licheniformis* ATCC 12759 in solid-state fermentation.](image)

![Figure 5: Effect of initial pH on β-galactosidase production by *B. licheniformis* ATCC 12759 in solid-state fermentation.](image)

| Agitation speed (rpm) | Specific activity (U/mg) |
|-----------------------|-------------------------|
| 60                    | 3175.3 ± 17             |
| 100                   | 3244.6 ± 12.1           |
| 120                   | 3266.6 ± 8             |
| 150                   | 3473.3 ± 12.7           |
| 180                   | 3065.2 ± 9             |
| 200                   | 2646.4 ± 12.1           |

(p < 0.05).
Table 3: Effect of medium volume on β-galactosidase production by B. licheniformis ATCC 12759 in solid-state fermentation.

| Medium size (mL) | Specific activity (U/mg) |
|-----------------|--------------------------|
| 100             | 3138.7 ± 13.7            |
| 250             | 3083.7 ± 8.7             |
| 500             | 2951.5 ± 20              |
| 1000            | 3032.7 ± 3.7             |

(p < 0.05).

Table 4: Carbon sources on the production of β-galactosidase by B. licheniformis ATCC 12759.

| Carbon source (1%) | Specific activity (U/mg) |
|--------------------|--------------------------|
| Control            | 3674.3 ± 78.3            |
| Sucrose            | 3083.3 ± 61.5            |
| Glucose            | 2903.2 ± 75.9            |
| Galactose          | 3524.7 ± 192.3           |
| Fructose           | 2880.3 ± 80.3            |
| Lactose            | 2928.8 ± 71.4            |
| Mannose            | 2968.4 ± 28.5            |
| Xylose             | 2690.6 ± 26.9            |
| Arabinose          | 3328.0 ± 33.2            |

(p < 0.05).

Apart from a good carbon source, RB also could serve as a nitrogen source, thus an increase in the complex nitrogen source adversely influenced the production of β-galactosidase.

Addition of metal salts source such as FeSO₄, MgSO₄, CuSO₄, ZnSO₄ and CaCl₂ to the medium were investigated. Comparison with the control (3475.6 U/mg), the production of β-galactosidase by B. licheniformis ATCC 12759 was suppressed when the bacterium was grown on metal salt sources (Table 6). Among the metal salt sources ZnSO₄ (52.1 U/mg) was greatly inhibited production of β-galactosidase by B. licheniformis ATCC 12759. On the other hand the salt requirement for production of this particular enzyme was apparently provided by the nature of RB.

Table 5: Nitrogen sources on the production of β-galactosidase by B. licheniformis ATCC 12759.

| Nitrogen source (1%) | Specific activity (U/mg) |
|---------------------|--------------------------|
| Control             | 3674.3 ± 63.0            |
| Sodium nitrate      | 2616.3 ± 35.9            |
| Ammonium sulfate    | 3200.4 ± 93.5            |
| Ammonium nitrate    | 2280.0 ± 73.5            |
| Ammonium chloride   | 1995.6 ± 63.6            |
| Beef extract        | 2110.4 ± 47.8            |
| Tryptone            | 3209.0 ± 104.0           |
| Peptone             | 1944.5 ± 90.5            |
| Yeast extract       | 1974.4 ± 121.5           |
| Urea                | 2735.8 ± 54.2            |
| Casein              | 1620.5 ± 61.5            |

(p < 0.05).

Discussion

Due to the potential usefulness of the β-galactosidase in the industrial applications, the development of methods for cheaper production of enzyme is very important. One alternative low cost production method is the use of SSF. In this study SSF has been found to be a cheap way of producing high levels of β-galactosidase by B. licheniformis ATCC 12759. The nature of solid substrate is the most important factor in SSF. This not only supplies the nutrients to the culture but also serves as an anchor for the microbial cells. An ideal solid substrate provides all necessary nutrients to the microorganism for optimum function [20]. Developing a β-galactosidase production process based upon RB as the solid substrate is very attractive, since it is a readily available source of carbon. It was previously reported that RB was found to be the best substrate for cellulase and β-glucosidase production by T. reesei and P. citrinum YS40-5 [21, 22]. This was in contrast to earlier report, which described wheat bran as a potential substrate for β-galactosidase production [23]. This is the first report of utilization of RB as solid substrate for β-galactosidase production by B. licheniformis ATCC 12759 by SSF. As a result was significant RB, further studies were carried out using this substrate.

The incubation time is governed by characteristics of the culture and also based on growth rate and enzyme production. The enzyme yield showed a gradual decrease on further extension of fermentation period which could be due to the depletion of nutrients for enzyme synthesis.
and proteolytic degradation of already synthesized enzymes [24].

The effect of temperature on β-galactosidase production is related to the growth of the organism. β-galactosidase yield increased with increase in incubation temperature from 30 to 37°C and higher temperature levels were detrimental to the growth and β-galactosidase production by B. licheniformis ATCC 12759, which may be attributed to the mesophilic nature of the microbe [24]. Maximum production at lower temperatures may be advantageous as it can reduce the rate of evaporation during incubation [20].

In SSF, optimum extraction of the product of interest from the fermented mass with a suitable solvent is a necessity. Parameters governing this process must therefore be established to maximize recovery [25]. From the results, it is clear that among all the solvents, tap water gave the best extraction of β-galactosidase from the fermented solids. This might be due to dissolution of the all fermented media by tap water which then becomes phosphate buffer and hence able to extract enzyme protein from fermented biomass. In contrast, distilled water is extractant (available, save and low cost) used for extraction of levansucrase from solid sawdust fermentation [26].

The inoculum level was also an important factor for the production of β-galactosidase. High inoculum levels are inhibitory in nature. A higher inoculum size may increase moisture content and lead to a decrease in growth and enzyme production; this may be due to the limiting nutrients at higher inoculum size and a lower inoculum size may require a longer time for fermentation to form the desired product [27–29].

The critical importance of moisture level in SSF media and its influence on the biosynthesis of enzymes has been attributed to the interference of moisture in the physical properties of solid particles [20]. The fact that the bacteria can grow and produce maximum β-galactosidase at lower moisture content of fermentable substrate offers significant advantages in reducing risk of contamination. Either low or high initial moisture significantly decreased the enzyme production. Lower moisture levels lead to reduced solubility of the nutrients, a lower degree of substrate swelling and higher water tension. Higher initial moisture in SSF decreases porosity, changes the particle structure and promotes development of stickiness due to agglomeration of the substrate. This reduces mass transfer process and gas exchange subsequently restricting the supply of oxygen for the growth of microorganism and leading to suboptimal product formation [24, 30].

Among the physicochemical parameters, the pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion [27]. Similarly, Dagbagli and Goksungur reported that pH 7.35 optimum for β-galactosidase production by Kluyveromyces lactis NRRL Y-8279 [28]. Results show that enzyme production was generally stable from pH 7.0, which indicates excellent buffering property of the agroresidues used for SSF [27].

To examine the effect of carbon source on the production of β-galactosidase B. licheniformis ATCC 12759, which produced a high amount of β-galactosidase with the highest specific activity (Table 1). The production of β-galactosidase by B. licheniformis ATCC 12759 was suppressed when the bacterium was grown on readily metabolizable sugars, since a low basal activity of β-galactosidase was detected in the culture medium. Several investigators have described the carbon source regulation of β-galactosidase biosynthesis in various microorganisms [31, 32]. All indicated that the role of carbon source in the biosynthesis of β-galactosidase may vary and depend on the microorganisms tested. Similarly, Konsoula and Kyriakides [33] reported the production of β-galactosidase by B. subtilis was greatly suppressed when the bacterium was grown on readily metabolizable sugars, since a very low basal activity of enzyme was detected in the culture medium in the presence of glucose, maltose, maltotriose or lactose. Kim and Rajagopal [34] described that addition of glucose or lactose to the growth medium inhibited the synthesis of β-galactosidase by L. crispatus. Easily metabolizable carbohydrates may result in the better growth of the bacteria along with reduction in the enzyme formation [35]. Although RB contains about 20% oil, 15% protein, and approximately 50% carbohydrate, of which starch is the main component, it has never been proposed as a source of sugars [36]. Since RB was used, carbon requirement could be met from it. This is important in terms of the cost of production of β-galactosidase.

Nitrogen source is an important factor that affects production of enzyme. Nizamuddin et al. [23] reported different organic and inorganic nitrogen sources improved the β-galactosidase production on RB medium produced by A. oryzae.

Agitation speed is a very important factor in the fermentation process since it will increase the amount of dissolved oxygen in the cultivation medium [37]. Agitation affects both air bubble dispersion and mixing of nutrients during fermentation process [38]. Dagbagli and Goksungur [28] reported that maximum β-galactosidase production from K. lactis NRRL Y-8279 was achieved when the optimal agitation speed of 179.2 rpm was used. Nadeem et al. [38] also found that the enzyme production was strongly affected by the agitation.
Laboratory-scale experiments may provide a basis for scale-up purposes of the process on β-galactosidase production in SSF [19]. Different types of production vessels have been used for carrying out solid state fermentation [39]; but most of the laboratory studies on the production of enzymes using SSF technique have employed Erlenmeyer flasks [19, 20, 23, 40]. The results were quite encouraging for the large scale production of the enzyme though the yields exhibited slight decline with the increase in substrate quantity which is probably due to lesser degree of aeration [20]. Future research will be focused on optimizing the nutrient conditions to obtain higher biomass, which is desirable for the industrial development of β-galactosidase product under low production cost [19].

Conclusion

In this study, we report the optimal cultivation conditions for β-galactosidase production by B. licheniformis ATCC 12759. Maximal amount of β-galactosidase production was obtained when SSF was carried out using RB, having inoculum level 35%, moisture content of 20%, initial pH 75 at 37°C for 48 h. Commercial β-galactosidase production is usually produced by SmF; however, SSF appear promising due to the natural potential and advantages they offer. Based on the present study, it appears that RB, which is inexpensive and readily available agricultural substance, could replace the commercial and more expensive substances in the development of a suitable economic fermentation medium for obtaining high yields β-galactosidase. However, the present study was entirely a laboratory-scale study, and it has to be further improved for a large-scale SSF.

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