Effect of nitrogen sources and fermentation conditions on bacillus sp. R2 chitinase production

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Effect of nitrogen sources and fermentation conditions on bacillus sp. R2 chitinase production

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Abstract

The nutritional and environmental conditions have a great influence on chitinase production, due to the receptor-inducer system that control enzymes production. In these sense, the aim of this study was to screen several nitrogen sources and optimize the most important fermentation conditions affecting Bacillus sp. R2 chitinase production. The results of one variable at time technique (OVAT) in shake flasks, revealed that among various nitrogen sources tested 0.5% yeast extract led to maximum production. Furthermore, the highest chitinase activity was detected after 24 h incubation period at temperature of 30°C, initial pH: 7.5, 2.5 to 3% NaCl concentration and under 180 rpm shaking using 2.5% (8.9 × 10⁸ CFU/ml) as best inoculum size. These optimizations can reduce the cost of chitinase production for the large scale specially

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Keywords: Bacillus sp. R2; nitrogen sources; chitinase; production conditions; OVAT; optimization.
1. Introduction

Chitin is the second most abundant biodegradable polymer, which exists naturally in the biosphere as a structural polysaccharide of β-1,4-N-acetyl-D-glucosamine. Chitin can be found as part of fungi, plants, crustaceans, insects, arthropods, and algae components [1]. Chitinases (E.C.3.2.1.14), is an important class of glycosyl hydrolases that play a key role in chitin decomposition and utilization as a renewable resource. These enzymes produced by a wide range of organisms including bacteria, actinomycetes, yeasts, fungi, plants, animals and human beings [2-3]. Bacteria produce chitinolytic enzymes for the assimilation of chitinous materials as carbon and nitrogen sources.

In microorganisms, chitinase production is controlled by a receptor-inducer system; therefore, the composition of the culture medium and fermentation conditions can affect significantly chitinase production [4].

During the last decade, Chitinases have drawn much attention in recent years due to their potential biotechnological applications. Besides the enormous application of chitinases in various fields, their commercial production and scale up is of critical importance, and are noticeably influenced by medium components and environmental factors [5,6,7], for this reason, the medium optimization studies and searching for chitinases production key factors, still an urgent need to maximize the production and meet the industrial demands.

In the previous studies, Bacillus sp. R2 was screened as hyper chitinase producer and the effect of Carbone sources on chitinase production was reported [8]. In the present work, the effect of nitrogen sources and fermentation conditions of chitinase production will be investigated using one variable at time technique (OVAT) in shake flasks.

2. Material and Methods

2.1. Chemicals

Chitin was extracted from crustaceans and squid by the method of (Synowiecki et al. 1982 [9], Crab shell chitin flakes (Win-lab, UK). Swollen chitin was prepared according to Monreal and Reese, (1969) [10]. Peptone tryptone, and yeast extract were obtained from (Oxoid Hampshire, England). N-acetyl glucosamine, and bovine serum albumin (BSA) were from (Sigma -USA), 2 Hydroxy 3,5 dinitrosalselic acid (DNSA) obtained from (Merck, Darmstadt- Germany). All other chemicals and reagents that were used were of highest grade commercially available.

2.2. bacterial strain cultivation and maintenance

Bacillus sp. R2 marine bacterial strain isolated from red sea Egypt and identified biochemically and molecularly by cheba et al 2006 (strain accession number in NCBI GenBank was: DQ923161). LB broth and peptone yeast agar medium(PYA) were used for cultivation. To maintain the isolated bacterial cells, the long-term maintenance was performed by adding 0.5 ml of the early stationary phase cultures grown in marine LB to 50% (v/v) sterile glycerol and the cultures were kept at-20°C.

2.3. Effect of nitrogen source on chitinase production

Sea water (SW) + 0.5 % colloidal chitin + 0.5 % glucose medium was supplemented separately with 0.5 % of one of the following inorganic and organic nitrogen sources: NH4Cl, NH4SO4, urea, tryptone, peptone, yeast extract and 0.25 % peptone + 0.25 % yeast extract. after 24 h incubation at temperature of 30C, chitinase activity was determined.
2.4. Optimization of chitinase fermentation conditions

The optimum conditions for chitinase production were determined using one variable at time technique (OVAT) in shake flasks (submerged fermentation). The tested factors were, sea water strength, NaCl concentration, pH, temperature, inoculum size and age, medium volume and agitation rate.

2.5. Chitinase assay

Chitinase activity was analysed according to the method of Miller (1959) [11] by estimating the released reducing sugars spectrophotometrically at 540nm. A standard curve was established prepared with a series of dilutions of N-acetyl – D-glucosamine (NAG) and DNSA. One unit of chitinase activity was defined as the amount of enzyme required to release 1 μ mol of NAG per minute during reaction conditions.

2.6. Protein content assay and Growth monitoring

Soluble proteins were determined as described by Bradford (1976) [12] using bovine serum albumin as standard. Colony forming units (CFU) was determined [13]. Moreover, bacterial growth was monitored spectrophotometrically by measuring the absorbance of the cultures at 660 nm.

3. Results and Discussion

3.1. Effect of nitrogen sources

Several organic and inorganic nitrogen sources were tested for the suitability to produce chitinase. Yeast extract was the most preferable source yielded the maximum chitinase specific activity. On the other hand, the lower activity was obtained in the presence of NH4Cl as a nitrogen source (Fig. 1). Data were in accordance with those of other researchers. [14-17].

![Fig. 1. Effect of nitrogen sources on chitinase production.](image-url)
3.2. Effect of yeast extracts concentration

Based on the above results, various concentrations of yeast extract were used in the medium to maximize the chitinase production. Yeast extract (0.5%) was the optimal concentration for highest chitinase specific activity. Concentrations higher than 0.5% exhibited a gradual decrease the chitinase activity (Fig. 2). Data agreed with those reported by many researchers. Yeast extract (0.5%) was the optimum for maximal chitinase production from *Bacillus circulans* [14], *Streptomyces cinereoruber* [18], *Enterobacter* sp. [19], *Cellulomonas flavigena* [20] and *Salinivibrio costicola* [21]. However, others reported that the range 0.01-2% of yeast extract was found to be the optimal concentration for chitinase production [22-25].

![Fig. 2. Effect of yeast extract concentration on chitinase production.](image1)

3.3. Optimization of chitinase fermentation conditions

3.3.1. Effect of sea water strength

Sea water strength plays an important role in cell growth and enzyme production concerning marine bacteria. It was noticed that from all tested sea water strength (dilutions) that 75% (v/v) sea water (Fig. 3) was the most appropriate dilution for achieving highest chitinase production. Results were in agreement with those reported by many investigators, sea water 75% was the best for chitinase production from *Vibrio alginolyticus* [26] and for agarase production from *Cytophaga* species. However sea water 50% was the optimal for chitinase and agarase production of *Beauveria bassiana* [27] and *Pseudomonas atlantica* [28], respectively. Generally, the range 50-100% of sea water was the preferable range for enzyme production and 50% was the least dilution that permitted the efficient growth for the marine microorganisms.

![Fig. 3. Effect of sea water strength on chitinase production.](image2)

3.3.2. Effect of NaCl concentration

Since most of the marine bacteria exhibit a broad tolerance to salinity [29] and our bacterium displays moderately halophilic or halotolerant properties, attempts were carried out to determine the suitable salinity level for chitinase
production. (Fig. 4) showed that the maximal chitinase activity was obtained at 2.5 to 3% NaCl concentration. Comparable results were obtained by Aunpad and Panbangrad 2003 NaCl (3%) was optimal for chitinase production by Salinivibrio costicola [21].

![Fig. 4. Effect of NaCl concentration on chitinase production.](image)

3.3.3. Effect of initial pH

The initial pH value of the medium has a significant effect on cell growth, cell membrane permeability, enzyme biosynthesis, secretion, activity and stability [30]. Accordingly, the effect of initial pH was examined. Results showed that, the pH has a bell shape effect (Fig. 5). The maximal chitinase activity was noted at initial pH 7.5. Most bacterial chitinases were produced at a pH range 6.5 – 8, while marine chitinases were produced optimally at pH more than 7 for example Altermonas sp. at pH 7.6-7.8 [23], Vibrio carchariae at pH 7.6 [31].

![Fig. 5. Effect of pH on chitinase production.](image)

3.3.4. Effect of temperature

Data presented in (Fig. 6) clearly indicated the influence of incubation temperature on chitinase production. The organism exhibited a good growth as well as enzyme production at 30°C. This temperature was optimal for chitinase production from marine bacteria [21-25-31], and for many terrestrial bacteria such as Bacillus circulans[14], B.cereus [32], B. pabuli [33], Enterobacter sp. [19], Cellulomonas flavigena [20], Pantoea dispersa [34], Streptomyces griseus [35] and S. albovinaceus [36].
3.3.5. Effect of inoculum size and age

Effect of inoculum size is a well-known factor in fermentation process. In the present investigation, there was a significant increase in chitinase production, growth rate and protein content with the increase in the inoculum concentration (Fig. 7). The best inoculum size was 2.5% (8.9 ×10^8 CFU/ml) for the optimal production. Further increase showed an adverse effect due to the short age of nutrients available for the larger biomass and faster growth of the culture. The optimal inoculum size reported ranged from 1-5% for example 1% was used for Cellulomonas flavigena [20] and Bacillus sp. NCTU2 [37] 2% for Bacillus sp. BG-11[38], Pantea dispersa [34]. The most appropriate inoculum age was 18 hrs (Table 1) where cells were fresh and very active for growth and enzyme production.

\[\text{Fig. 6. Effect of temperature on chitinase production.}\]

3.3.6. Effect of medium volume and agitation rate

Since our microorganism was aerobic, the effect of medium volume and agitation speed on chitinase yield were considered. Data of. (Table 1) demonstrated that 50ml/250ml flask and 180 rpm were the best medium volume and
agitation speed, respectively. Chitinase production from *Trichoderma harzianum* was increased with increasing the agitation rate between 150 and 300 rpm [39]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Chitinase specific activity (U/mg)</th>
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<td>Inoculum age (h)</td>
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</table>

4. Conclusion

The main conclusion drawn from the present study indicated that the optimization of medium composition of nitrogen sources and fermentation conditions affect significantly chitinase production and may played a pivotal role in cost reduction for the large scale production specially.

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References


