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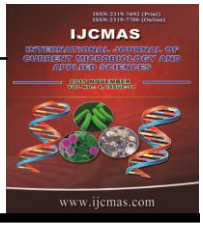

Original Research Article

Optimization of Cultural Conditions for Production of Chitinase by *Bacillus* sp. Isolated from Agriculture Soil using Substrate as Marine Crab Shell Waste

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A B S T R A C T

Keywords

Crustacean, Chitinase, *Bacillus* sp.

The chitinase production with crustacean waste powder at different concentrations of substrate (0.5, 1.0 and 1.5%) was increasing. Among the two forms of substrate with three different concentrations, the combined substrate (crustacean waste powder and colloidal chitin) of 1% was found to be the best substrate for optimal chitinase production by *Bacillus* sp. Subsequently, the optimal production with such substrate was achieved on pH8. For further work, it is necessary to over express the enzyme in the suitable production, which was followed by the purification and characterization of the chitinase in order to know its properties.

Introduction

Chitin is a polysaccharide, a linear polymer composed of N-acetyloglucosamine monomers connected by glycoside β - 1, 4 bonds. It forms an outer skeleton of arthropods, protozoa, molluscs, nematoda, crustaceans and is present in the cell walls of many fungi. Biological degradation of chitin is accompanied by endo- and exo- enzymes known as chitinases and β-N-acetylohexaminidases. Enzymes participating in chitin degradation are produced not only by organisms containing chitin in their body but by bacteria, upper plants and mammals where chitin is not present on a regular basis. Bacteria produced chitinases play a significant role in maintaining the matter cycle through making chitin usable biologically. The production of high added-value products, such as, chitin, chitosan and their derivatives along with their application in different fields are of utmost interest (Shahidi *et al.*, 1999). In our present study to optimization of cultural conditions for production of chitinase by *Bacillus Sp* isolated from agriculture soil using substrate as marine crab shell waste

Materials and Methods

The soil sample were collected from the agricultural field and isolate the bacterial strains from the samples by serial dilution method (R.C.Dubey and D.K.Maheshwari., 2002) using the plates from the above experiment, observe the colony draw and name the colony draw and identified the Microorganism by staining method.

Biochemical characterization (IMVIC Test)

Identification of selected isolate was studied based on different morphological, physiological and biochemical characteristics. The purified bacterial culture was subjected to a range of biochemical tests for identification.

Preparation of chitin substrates for enzyme assay

In our experiment, chitinase production was observed towards the substrate, marine crab shell waste powder as well as its combination with colloidal chitin. Each substrate was prepared at three following concentrations: 0.5%, 1.0% and 1.5%.

Preparation of chitin powder from marine crab shell waste

The crustacean waste powder used in this experiment was prepared from crabs waste. Preparation of the crustacean waste powder was carried out according to the method modified by Oktavia *et al.* (2005). Colloidal chitin was made from commercially available chitin powder (Sigma) based on the method as described by Arnold and Solomon (1986).

Optimization of chitinase production

Production of chitinase from the bacterium was performed by growing it at 55°C in the 150-ml broth Minimal Synthetic Medium (MSM) containing 0.1% $2HPO_4$, 0.01% $MgSO_4.7H_2O$, 0.1% NaCl, 0.7% $(NH_4)_2SO_4$, 0.05% yeast extract. Then the bacterial cultivation was carried out with crustacean waste powder at different concentrations (0.5, 1.0, and 1.5) and then culture sampling was conducted regularly everyday for 6 days. In addition, chitinase production was also observed towards the mixture of colloidal chitin and crustacean waste powder.

Extraction of chitinase

Isolate extracellular chitinase, 10-ml cell culture was centrifuged at 10.000 rpm, 4°C for 25 minutes. The resulting cell-free supernatant was used further for activity assay. Chitinase activity was determined by a dinitrosalicylic acid (DNS) method (Miller, 1959). This method works on the concentration of *N*-acetyl glucosamine (NAG), which is released as a result of enzymic action.

Influence of temperature on crude extract

The minimal synthetic medium was prepared for the optimization condition under different temperature (17°C, 27°C, 37°C, 47°C and 57°C). The minimal synthetic medium was prepared and the marine bacillus *sp* (inoculum) were inoculated and incubated at different temperature of (17°C, 27°C, 37°C, 47°C and 57°C) after incubation the crude were extracted and tested for antimicrobial activity (National Committee for Clinical Laboratory Standards, 1997).

Influence of other factors on crude extract

The minimal synthetic medium was prepared for the optimization condition under different pH (5, 6, 7, 8 and 9) Carbon and nitrogen sources. The *bacillus sp* (inoculum) was inoculated and incubated at optimized temperature of 47°C. After incubation the crude were extracted and tested for antimicrobial activity.

Partial purification of enzyme

Purification steps were carried out with the

help of two different methods of ammonium sulphate precipitation and ion-exchange chromatography. The eluted samples were assayed for enzyme activity and separate of protein and estimate molecular weight by SDS- PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) and preserved enzyme by immobilization method (Meena and Raja, 2004)

Results and Discussion

Isolation of bacteria (*Bacillus spp.*)

The soil sample was serially diluted and spread into the nutrient agar medium. After incubation period the colonies were counted and the dominated colonies were picked out and their morphological characterization was studied. The dominated colonies were streaked and the pure culture was stored for further studies. Based on morphological, microscopical and biochemical characterization, the bacterium was identified as *Bacillus subtilis* and *Bacillus cereus*

Preparation of chitin substrates for enzyme assay

In our experiment, chitinase production was observed towards the substrate, marine crab shell waste powder as well as its combination with colloidal chitin. Each substrate was prepared at three following concentrations: 0.5%, 1.0%, and 1.5%. The chitinase production was well good in the percentage of 1.5% concentration (Table 1).

Effect of pH

The effect of initial pH of the medium on chitinase production was studied by adjusting the initial pH from 6 to 10 by using 0.1N HCl- NaOH. The optimization of cultivation condition for the production of chitinase with different pH was carried out. The best pH for the production of chitinase was shown in table 2.

Effect of temperature

The effect of temperature of the medium on chitinase production was studied by altering the incubation temperature. The temperature was adjusted in the range from 17°C to 57°C. The best temperature for the production of chitinase was shown in table 3.

Effect of carbon source in different concentration

Carbohydrate utilization by bacteria for the production of chitinase enzyme in aerobic fermentation was studied. Different carbon sources were used in 50ml level in the cultivation medium (Glucose, sucrose, lactose, maltose and fructose). The optimization of its cultivation conditions for the chitinase producing with different carbon source. The best carbon source for the production of chitinase, the activity was shown in table 4.

Effect of nitrogen source in different concentration

Nitrogen utilization by bacteria for the production of chitinase enzyme in aerobic fermentation was studied. Different nitrogen sources in 50ml level in the cultivation medium.

The optimization of its cultivation conditions for the chitinase producing with Nitrogen source in different concentration. The best nitrogen source in different concentration for the production of chitinase activity was shown table 5.

Optimization of culture conditions

Chitinase produced by *Bacillus subtilis and Bacillus cereus* were incubated under all optimal nutritional and environmental fermentation conditions. These properties include: effect of pH, effect of temperature, effect of carbon source in different concentration, effect of nitrogen source in different concentration (Table 6).

Purification

The extracted sample was determined activity of enzyme initial treatment of Ammonium salt with stirring**.** The dialyzed sample was purified with DEAE (Di Ethyl Amino Ethyl cellulose) column**.** The eluted samples were collected and assayed chitinase enzyme activity, protein assay (Table 7). This molecular mass was confirmed by single band in SDS-PAGE. The protein is a monomeric had a molecular mass of *Bacillus subtilis* and *Bacillus cereus,* The produced enzymes were entrapped with Sodium alginate and Calcium chloride**.**

Optimization of chitinase production was carried out by cultivating *Bacillus* sp. in the media containing crustacean waste powder at three different concentrations (0.5, 1.0, and 1.5%). In addition to that, chitinase production was also observed towards the mixture between crustacean waste powders and colloidal chitin at such three different concentrations. It was assumed that the continuous production of chitinase was due to the enough availability of chitin in the growth medium, as the result of the slow rate of chitin biodegradation during the cultivation. Subsequently, the slow rate is probably due to the low solubility of crustacean waste powder in the medium.

However, the chitinase production in our experiment was probably induced by the chitin added into the growth medium. It was assumed that chitin and its degradation products played a role as an inducer system in stimulating the production of chitinase, as reported (Felse and Panda, 1999). This case was also similar to that reported by Frandberg and Schnurer (1994) that the products of chitin degradation, mainly *N,N'*-diacetylchitobiose (GlcNAc2), induced the chitinase production by *Bacillus pabuli*. Especially when chitin is added into the medium, it is partially hydrolyzed by extracellular chitinases into oligomers, mainly dimers of GlcNAc (GlcNAc2), which is hydrolyzed further by β-*N* acetylglucosaminidase (GlcNAcase) to form the monomers, GlcNAc. Then these monomers are taken up via the phosphotransferase system (Park *et al.*, 2000).

The chitinase production with the combined substrate (crustacean waste powder and colloidal chitin) at the concentration of 1.0 and 1.5% showed that the highest activity was achieved indicating that the optimal production of chitinase was on combination of the colloidal chitin and crab waste chitin.

This subsequently suggests that the presence of colloidal chitin in the growth medium could increase the chitinase production, which significantly appeared. Subsequently, it was found that the activity at the lower concentration (0.5%) was a bit higher than that at the higher ones (1.0 and1.5%). It appears that it took a longer time for chitinase to degrade the chitin in the waste powder, due to the low solubility of the powder in the growth medium, but the production of chitinase is very high in the concentration of 1.5%.

Table.1 Chitinase assay crude (different substrate in different concentration)

S.No	Concentration of substrate in percentage (synthetic chitin and crab waste powder-1.5%) (u/ml)		
	0.5	1	1.5
Sample1	34.34	45.24	62.45
Sample2	34.06	43.89	60.19
Sample3	32.00	42.26	61.02

Table.2 Chitinase assay on different pH

S.No	Chitinase production on different pH(synthetic chitin and crab waste powder -1.5%) (u/ml)				
	6	7	8	9	10
Sample1	46.07	62.43	76.59	49.21	56.26
Sample2	43.79	61.30	72.09	42.68	52.80
Sample3	43.22	63.00	75.00	48.87	49.89

Table.3 Chitinase assay on different temperature

S.No	Chitinase production on different temperature (synthetic chitin and crab waste powder -1.5%) (u/ml)				
	17°C	27°C	37°C	47°C	57°C
Sample1	62.30	80.21	96.59	55.36	51.28
Sample2	59.84	79.20	95.34	47.18	48.10
Sample3	60.52	81.02	93.64	52.55	51.00

Table.4 Chitinase assay on different carbon source

S.NO	Chitinase production on different carbon source (synthetic chitin and crab waste powder -1.5%) (u/ml)				
	GLUCOSE	LACTOSE	FRUCTOSE	SUCROSE	MALTOSE
Sample1	51.78	63.54	80.21	97.06	52.57
Sample2	49.60	61.08	72.13	96.32	51.90
Sample3	50.64	60.88	72.15	90.23	54.01

Table.5 Chitinase assay on different nitrogen source

S.No	Chitinase production on different nitrogen source (synthetic chitin and crab waste powder -1.5%) (u/ml)				
	Beef extract	Yeast extract	Peptone	Sodium nitrate	Ammonium nitrate
Sample1	80.35	92.21	72.59	53.21	66.76
Sample2	79.98	91.30	70.79	50.68	62.43
Sample3	81.65	91.55	68.56	56.41	62.00

Table.6 Optimized production medium for chitinase assay

Organisms	P^H	Temperature (°c)	Carbon source in different concentration (sucrose) (g/ 50ml)	Nitrogen source in different concentration (yeast extract) (g/ 50ml)	Enzyme concentration (u/ml)
Sample1	8	37°C	1.50	0.25	**192.64**
Sample2	8	37°C	1.50	0.25	**186.21**
Sample3	8	37°C	1.50	0.25	**187.00**

Table.7 Chitinase assay for purification

Contents	Enzyme concentration (u/ml) by salt cut	Enzyme concentration (u/ml) by ion-exchange
Sample1	197.11	196.56
Sample2	190.86	193.12
Sample3	190.50	194.00

The phenomenon was different from that on the combination of colloidal chitin and crustacean waste. The optimal specific activity for this combined substrate (0.5, 1.0 and 1.5%) was achieved.

Among the two species Bacillus species, the *bacillus subtilis* possess the high chitinase production 62.45 which was shown in the table 2. The optimization of the medium with the pH of the 8, temperature 37°C, carbon source-sucrose, nitrogen source – yeast extract. Similarly, the chitinase production in our experiment was probably induced by the chitin added into the growth medium. It was assumed that chitin and its degradation products played a role as an inducer system in stimulating the production of chitinase, as reported (Felse and Panda, 1999). This case was also similar to that reported by Frandberg and Schnurer (1994) that the products of chitin degradation, mainly *N,N'*- diacetylchitobiose (GlcNAc2), induced the chitinase production by *Bacillus pabuli*.

The pH of the culture medium is playing important role in chitinase production. Majority of the bacteria reported to produce maximum level of chitinase at neutral or slightly acidic pH and whereas fungi mostly secret it in acidic conditions (Sharaf, 2005).

Analysis of the results has revealed that the potential of any microbial culture could be increased for the production of different metabolites by adapting suitable culture techniques. The partially purified enzyme shows the more activity. The purified enzymes were preserved by immobilization. Our results are in line with findings provided by the ammonium hydroxide fraction. It also confirms that this system of purification is reproducible, as the pattern of SDS-PAGE was nearly the same when the experiment was repeated.

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