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Effect of Carbon Sources on *Bacillus sp.* R2 Chitinase Production

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ABSTRACT

Effect of carbon sources on Bacillus sp. r2 chitinase production were determined using one variable at time technique (OVAT) in shake flasks (submerged fermentation). The tested factors were chitin sources and forms, colloidal chitin concentration, secondary carbon sources and glucose concentration. The highest chitinase activity (57 U/ml) was obtained in 75% natural sea water, 0.5% shrimp shell colloidal chitin as best and essential carbon source, 0.5% glucose. The production also was optimum medium volume 50 ml/250ml flasks and 24 h incubation period at 180 rpm shaking. carbon sources affect strangely enzyme induction and may help to understand the physiology of enzyme production

KEYWORDS: *Bacillus sp. R2, Carbon Sources chitinase, production, OVAT, optimization.*

INTRODUCTION

Chitin, a β- (1-4) homopolymer of N-acetyl - D- glucosamine (Glc NAc), is the second most abundant polysaccharide existing in nature after cellulose. It is a major structural component of most biological systems such as mollusks, insects, crustaceans, fungi, algae and marine invertebrates [1]. Chitin and its derivatives are of commercial and biotechnological interest because they have various biological activities and wide range of applications in areas ranging from waste water treatment to agrochemical and biomedical uses [2,3,4,5]. Chitinases (E.C.3.2.1.14), which are found in variety of organisms such as viruses, bacteria, actinomycetes, yeasts, fungi, plants, animals and also in human beings [6,7]. During the last decade, chitinases have received an increased attention due to their wider ranges of biotechnological applications especially in the biocontrol of fungal phytopathogens [8] and harmful insects [9]. They have also been used as vaccine [10], antitumor [11], tumor marker [12] and useful biomarker in Gaucher disease [13] and used in the preparation of sphaeroplasts and protoplasts from yeasts and fungal species which can be used further for biotechnological uses [14]. In the previous studies, Bacillus sp. R2 was screened as hyper chitinase producer and identified. In the present work the effect of carbon sources on Bacillus sp.r2 chitinase production will be determined using one variable at time technique (OVAT) (OVAT) in shake flasks.

MATERIAL AND METHODS

Substrates and chemicals:

Chitin was extracted from crustaceans by the method of [15], Crab shell chitin flakes (Win-lab, UK). Swollen chitin was prepared according Monreal and Reese, [16]. Peptone tryptone, and yeast extract were obtained from (Oxöid Hampshire, England). 2 Hydroxy 3,5 dinitrosalselic acid (DNSA) obtained from (Merck, Darmstadt- Germany); N-acetyl glucosamine, agarose, bovine serum albumin (BSA) were from (Sigma -USA). All other chemicals used were of the highest grade available.

Bacterial strain and culture condition:

Bacillus sp. R2 was newly marine bacterial strain isolated from red sea Egypt, identified either by biochemical tests or 16S rRNA methodology (strain accession number in NCBI GenBank was: DQ923161). To maintain the isolated bacterial cells the short-term maintenance was performed repeatedly at an interval of 2-3 months at 4°C using marine LB Agar slants. Moreover, the long-term maintenance, more than 2 years, 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.For chitinase production several single colonies of the strain Bacillus sp. R2 were used to inoculate 5 ml marine LB medium supplemented with 0.5% colloidal chitin. This bacterial culture could grow at 37ºC for 24 h using orbital shaking incubator, Overnight seed culture 2,5% (v/v) was used to inoculate 50 ml production medium. the culture was incubated at 30ºC for 24 h at 180 rpm shaking.

Optimization of chitinase production:

Effect of carbon sources on Bacillus sp.r2 chitinase production were determined using one variable at time technique (OVAT) in shake flasks (submerged fermentation) .The tested factors were chitin sources and forms, colloidal chitin concentration, secondary carbon sources and glucose concentration.

Analytical procedures:

Growth monitoring and Protein assay:

Colony forming units (CFU) was determined [17]. Moreover, bacterial growth was monitored spectrophotometrically by measuring the absorbance of the cultures at 660 nm. Soluble proteins were determined as described by Bradford [18] using bovine serum albumin as standard

Chitinase assay:

Chitinase activity was analyzed by estimating the released reducing ends of sugar according to the method of Miller [19] using N-acetyl - D-glucosamine (NAG) as standard. One unit of chitinase activity was defined as the amount of enzyme required to release 1 µ mol of NAG per minute during these conditions.

RESULTS AND DISCUSSION

Optimization of chitinase production:

Effect of chitin sources and forms:

Chitinase production was remarkably affected by the nature and form of chitin. The shrimp shell colloidal chitin was the best substrate followed by Sigma crab shell chitosan and prawn shell colloidal chitin powder (Table1). Chitin flasks were not as effective as colloidal chitin. This may be due to the open structure of colloidal chitin which makes it more accessible by the enzyme. Results agreed with those reported about chitinase inducibility by colloidal chitin [20,21,22,23]. These chitins still vary however in strand opening (degree of compactness), degree of acetylation and probably the presence of covalently linked components other than N-acetyl glucosamine.

Effect of colloidal chitin concentration:

It is known that an ideal substrate concentration in any fermentation process results in a higher conversion efficiency and an optimum substrate utilization [24]. Based on above results Figure (1) colloidal chitin was the most suitable substrate for chitinase production. Various concentrations of colloidal chitin were tested in order to obtain the maximum chitinase production, the optimum concentration for chitinase production was 0.5%. Many investigators reported that the optimal concentration ranged between 0.5 and 1 %[25, 22,26,27].

Effect of carbon sources:

Various carbon source supplementations were tested to investigate their effect on the chitinase production. Glucose, N-acetylglucosamine, maltose and pectin showed significant increase in chitinase production based on units/mg protein, respectively (Table 2). On the other hand, glycerol, Tween 20 and Tween 80 gave only maximum growth rate and protein content. Other carbon sources did not promote the enzyme production. Many

workers found that, the addition of glucose, glucosamine or N-acetylglucosamine, stimulated chitinase production. [28,29,30,22,31,32] Moreover, others reported that, the addition of maltose [33], β -cyclodextrin [34], pectin (35) or CMC (36) also enhanced significantly the chitinase production.

Effect of glucose concentration:

Since the addition of glucose stimulates the chitinase production, attempts were made to determine the suitable glucose concentration. The best concentration of glucose was found to be 0.5% which gave maximum induction level after 24 hrs. incubation. However, concentrations more than 0.5% showed an adverse effect (Figure 2). It was reported that the optimal glucose concentration for chitinase production ranged between 0.2 and 1%. It was 0.2% for *Acinetedacter sp* [30], 0.3% for *Aspergillus sp.* [25], 0.5% for *Bacillus sp.*BG11 [31] and *B. licheniformis*[22], 0.6% for *Vibrio alginolyticus*[28] and 1% for *Salinivibrio costicola*[29]. Furthermore, it was noticed for marine bacteria that glucose, trehalose and chitin hydrolysis end products e.g., N-acetyl glucosamine, glucosamine and chitobiose play an important role in chemotaxis and induce the chitinase production [37,38,39].

Table 1: Effect of chitin sources and forms on chitinase production by strain R2.

Substrate sources	Substrate forms	Protein (mg/ml)	Activity (U/ml)	Specific Activity (U/mg)
Crab shell chitin-flasks (Win lab.UK)	α	0.095	9.66	101.4
Crab shell chitin-powder (Winlab.UK)	α	0.105	11.4	108
Crab shell chitin	α	0.081	9.32	115
Shrimp shell chitin	α	0.060	8	131.9
Prawn shell chitin	α	0.071	10.3	143.8
Squid chitin	β	0.075	14.2	189.3
Crab shell colloidal chitin (Win lab)	α	0.125	24.1	191.5
Crab shell swollen chitin	α	0.129	21.2	163.7
Crab shell colloidal chitin	α	0.118	20.6	174.4
Shrimp shell colloidal chitin	α	0.133	31.3	234.1
Prawn shell colloidal chitin	α	0.153	32	208.2
Crab shell chitosan (Sigma - USA)	α	0.103	22.7	219.5
Crab shell chitosan	α	0.106	19.5	182.6
Crab shell colloidal chitosan	α	0.131	13	98.5

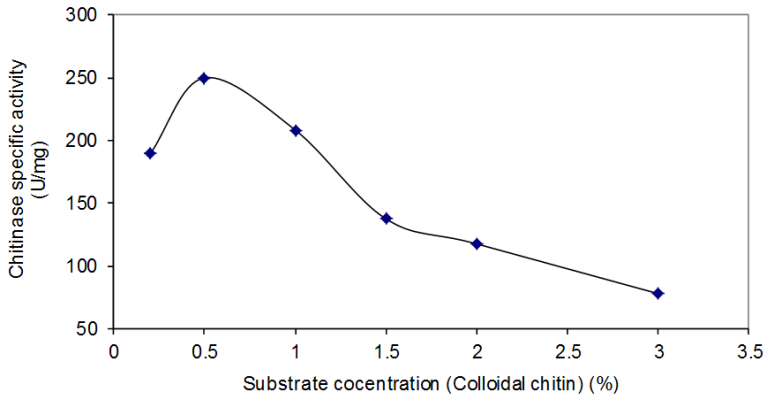

Fig. 1: Effect of substrate concentration on chitinase production

Table 2: Effect of carbon source on chitinase production

Carbon source	Conc. (%)	Growth OD_{660nm}	Chitinase activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)
Monosaccharides					
Glucose	0.5	1.94	48.02	0.115	417
Galactose	0.5	1.87	14.61	0.143	102.16
Fructose	0.5	1.94	5.25	0.117	44.87
Sorbose	0.5	1.52	12.86	0.129	99.6
Arabinose	0.5	1.93	21.21	0.132	160.68
Xylose	0.5	1.92	2.42	0.112	21.60
Mannose	0.5	1.68	0.53	0.096	5.52
Disaccharides					
Maltose	0.5	1.89	37.91	0.122	310.77
Lactose	0.5	1.81	12.45	0.091	136.81
Sucrose	0.5	1.78	7	0.089	78.65

Amino sugar (NAc glucosamine)	0.5	1.93	41.41	0.108	383.42
polyols (glycerol)	0.5	2.23	6.12	0.105	58.28
Glucose based polysaccharides					
Starch	0.2	1.91	36.76	0.124	296
Dextran	0.2	1.75	0.134	0.071	1.88
CMC	0.2	1.64	2.02	0.062	32.58
Cellulose	0.2	1.52	1.75	0.053	33.01
Galactose based polysaccharides					
Agar	0.1	1.73	6.80	0.078	87.17
Agarose	0.1	1.85	10.23	0.081	126.29
Pectin	0.2	1.87	30.43	0.104	292,.59
Arabic gum	0.2	1.61	8.48	0.075	113.06
Miscellaneous					
Alginate	0.2	1.57	13.67	0.087	157.12
Tween 20	0.2	2.36	7.81	0.238	32.81
Tween 80	0.2	2.58	10.16	0.250	40.64

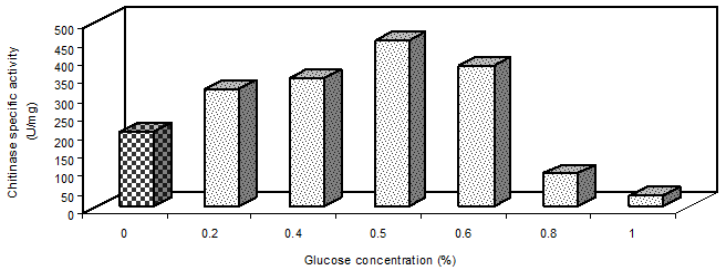

Fig. 2: Effect of glucose concentration on chitinase production

Conclusion:

The optimization of medium composition specially carbon sources which affect strangely enzyme induction and plays a crucial role in the microbial production of chitinases and help to understand the physiology of enzyme production, recognizing the key factors, setting the best bioprocess (fermentation) conditions which pave the way for the industrial scale-up with low cost and high enzymatic yield.

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