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Demineralized crab and shrimp shell powder: Cost effective medium for bacillus Sp. R2 growth and chitinase production

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Abstract

Crustaceous wastes such as shrimp, crab and lobster, are chitinous solid waste of the shellfish processing industry. to select the best substrates for bacterial growth and enzyme activity, different poor and enriched media were investigated, furthermore, the non-dematerialized and dematerialized shells powders of crab, shrimp, prawn and the mixed powders of crab+ shrimp shells and crab+ prawn shells were tested as substrates for and chitinase production by the chitin degrading bacterium Bacillus sp. R2 in a submerged fermentation (SMF) culture. The results revealed that, the tested poor media failed to enhance enzyme production, whereas the tested enriched media promote growth and protein content efficiently, in contrast the demineralized chitinous wastes were generally good carbon and nitrogen sources for growth and chitinase production. The most favorable substrates were demineralized crab + prawn followed by crab + shrimp. The obtained results would certainly encourage the utilization of shellfish processing (Crab and Shrimp Shell) waste for the industrial production of chitinase via submerged fermentation (SMF).

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Keywords: Bacillus sp. R2; chitinous waste; crab and shrimp shell; chitinase; production.

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1. Introduction

It is reported that approximately 6-8 million tons of crustacean waste is produced worldwide every year [1]. The current increase in crustacean wastes from shrimp and crab industry in the most producing countries in the word (China, Indonesia, Thailand and India) pose serious disposal problems, bioconversion of crustacean waste has been proposed as an alternative treatment [2]. During the last decade, chitin-containing marine crustacean waste have received an increased attention since the major components of this waste are chitin, protein, flavorant, pigment and minerals. The bioprocessing using chitinolytic microorganisms or their chitinases not only resolves the environmental problem but also ensure the full utilization. for this reason, the biological method (bacteria or enzymes) are preferable because it is economic, safe, eco-friendly and can maximize the crustacean wastes utilizations such as the recovery of pigment, lipids, chitin, chitosan, chitooligosaccharides and the protein hydrolysate which can be used as an excellent fish or animal feed. [3,4]

Recently many researchers investigated the bioconversion of shrimp and crab shell powder (SCSP) for the production of many valuable compounds such as proteases[3], chitinases[4], chitosanases ,antifungals and antimicrobial compounds[5] ,antioxidants[6], carotenoids, astaxanthin[7], flavour compounds, and calcium carbonate[8]. The objective of this research was to investigate the suitability of some treated and untreated chitinous wastes as cost effective media for *Bacillus sp. R2* growth and multiple enzymes production.

2. Material and Methods

2.1. Chemicals

Chitin was extracted from crustaceans by the method of (Synowiecki *et al*. 1982) [9], Swollen chitin was prepared according Monreal and Reese, (1969) [10]. Crab shell chitin flakes (Win-lab, UK). N-acetyl glucosamine, and bovine serum albumin were from (Sigma -USA). Peptone tryptone, and yeast extract were obtained from (Oxöid Hampshire, England). All other chemicals used were of the highest grade available.

2.2. Crustaceous wastes preparation and demineralization

Raw dried offal's or shells of crab, shrimp, prawn, squilla, squid and clams were milled as fine as possible and demineralized with 22% HCl (1:10) (w/v) for 2 hours at room temperature with vigorous steering [9].

2.3. Microorganism 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). 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.

2.4. Effect of poor and enriched media on chitinase production

Chitinase activity and protein content were determined through growing of the bacterial isolate in different commonly used liquid media. The poor media were: (Natural sea water + 0.05 % yeast extract), (Artificial sea water + 0.05 % yeast extract), M9 minimal medium and William Basel medium, the enriched media were: LB medium, PY medium, Nutrient broth (NB) and marine Nutrient broth (M NB).

2.5. Effect of chitinous wastes on cell growth and enzymes production

Sea water medium (100% sea water) pH: 7.5 were supplemented separately with 0.5% of non-demineralized or demineralized chitinous wastes. After autoclaving, the flasks were inoculated with 3% of overnight activated strain

and cultures were incubated for 24 h at 30ºC and 150 rpm. The tested non-dematerialized and dematerialized chitinous wastes were the shells powders of crab, shrimp, prawn and the mixed powders of crab+shrimp shells and crab+ prawn shells.

2.6. Analytical procedures

2.6.1. Protein content assay and Growth monitoring

The soluble proteins were determined as described by Bradford (1976) [11] using bovine serum albumin as standard. Colony forming units (CFU) was determined [12]. Moreover, bacterial growth was monitored spectrophotometrically by measuring the absorbance of the cultures at 660 nm.

2.6.2. Chitinase assay

Chitinase activity was analysed according to the method of Miller (1959) [13] 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.

2.6.3. Protease assay

Proteolytic activity (neutral and alkaline) was measured according to the method of Cliffe and Law (1982) [14] using Hide Powder Azure (HPA). One unit of proteolytic activity was defined as the amount of enzyme that developed a change of absorbance (0.1) against the control at 595nm at 37°C.

2.6.4. Lipase assay

Lipolytic or esterolytic activity was determined according to the method described earlier using *P*- nitro phenyl palmitate as substrate [15]. One unit of lipolytic or esterolytic activity was defined as the amount of enzyme that released one micromole of *P*- nitro phenyl from the substrate *P*- nitro phenyl palmitate per min.

3. Results and Discussion

3.1. Effect of incubation period

Chitinase activity, O.D at 660 nm and protein content of growing culture directed by strain R2 were determined each 12 hrs for 4 days. The maximum enzyme activity was obtained in 24 hrs; however, it was dropped gradually there after (Table 1). These results agreed with other marine bacteria, that produce chitinase within just one day such as Alteromonas sp. [16], Vibrio alginolyticus [17], Salinivibrio costicola [18] and Bacillus sp. MET 1299[19].

3.2. Effect of poor and enriched media

Four poor laboratory media and other four enriched laboratory media were tested for their ability to support chitinase production by the strain R2. The natural sea water medium gave the highest chitinase specific activity (Table 2). However, these results were still very low when compared with that obtained in the presence of colloidal chitin. Data were consistent with the fact that most chitinases were inducible enzymes. Other tested rich media supported the bacterial growth and the protein content; however, they did not support the enzyme production (Table 2).

3.3. Effect of chitinous wastes as carbon source

A preliminary experiment was carried out to test the suitability of some chitinous wastes (as substrates for strain R2) for chitinase production. Data revealed that, the demineralized chitinous wastes were generally good carbon and nitrogen sources for growth and chitinase production. The most favorable substrates were demineralized crab +

prawn (242.6 U/ mg) and crab + shrimp (218.70 U/ mg), respectively (Fig. 1). These results were in agreement with those reported by many researchers [20-22]. Recently, Wang *et al.* 2006[23] and Chang *et al.* 2007 [24] produced chitinases from *Bacillus subtilis* W-118 and *B. cereus* QQ308 when grown on crab and shrimp shell powder as major carbon source, respectively.

Table 1. Effect of incubation period on chitinase production.

Incubation period (h)	Chitinase activity (U/ml)	Growth (OD_{660} nm)	Protein content (mg/ml)	pH (initial: pH;7.5)
12	15.55	0.731	0.061	
24	31.20	1.78	0.083	7.80
36	22.4	1.82	0.096	
48	16.3	1.85	0.081	8.4
60	11.5	1.72	0.052	
72	5.2	1.53	0.043	8.2
96	1.3	1.44	0.048	8

Table 2. Effect of poor and enriched laboratory media on chitinase production.

Medium + 0.5% C.CH	Chitinase activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)
Poor media:			
• Natural sea water + 0.05 Y. E	8.84	0.071	124.5
• Artificial sea water + 0.05 Y.E.	6.12	0.095	64.42
• M_9 minimal medium	5.58	0.074	75.42
• William basal medium	4.44	0.067	66.26
Enriched Media:			
• Lauria and Bertani (L.B)	11.58	0.135	85.77
• Peptone yeast medium (PY)	8.35	0.174	47.9
• Nutrient broth (N.B)	6.12	0.261	23.44
• Marine (N.B): (S. W +N.B)	10.16	0.203	50.09

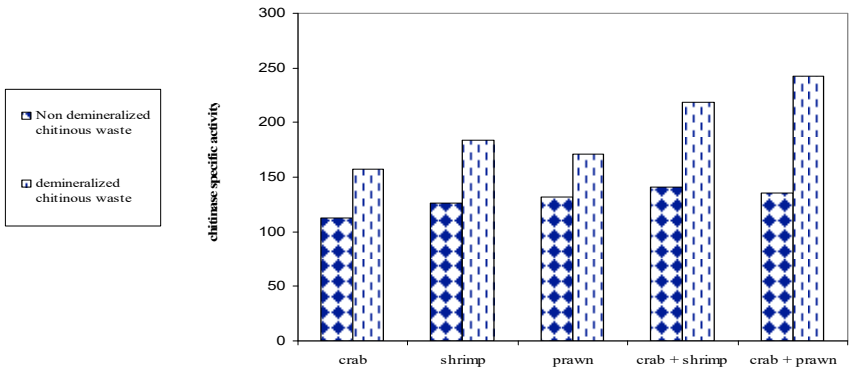

Fig. 1. Effect of some chitinous wastes on chitinase production.

3.4. Chitinous wastes for cell growth and enzymes production

Since the chitin in the shell fish wastes was tightly associated with proteins, inorganic salts such as calcium carbonate, and lipids including pigments [25]. Thus, proteases and lipases together with chitinases could be necessary for efficient degradation of crustacean cuticles in the natural environment, and since our bacterium was chitinolytic, proteolytic and lipolytic, a series of experiments were designed and conducted with the aim to utilize and recycle these chitins containing wastes. Data registered in Table (3) demonstrated that the non - demineralized chitinous wastes in general were considered the best carbon and nitrogen sources for growth and lipase production. For example, the highest growth rate (1.28) and lipase activity (342, 9U/ml) after only one-day fermentation were obtained with non - demineralized prawn shell.

Table 3. Effect of chitinous wastes on cell growth and enzymes production.

	Chitinous waste (2%)	Growth	Deproteination folds	enzyme activity (U/ml)		
		OD at 660nm		Chitinase	Protease*	Lipase
Crude wastes	Crab	0.613	0.55	21.17	1.25	-
	Shrimp	0.981	0.78	23.76	1.58	-
	Prawn	1.28	0.78	31.18	1.35	342.9
	Squilla	0.789	0.62	25.38	1.21	-
	Squid	0.371	1.77	10.02	0.970	-
	Clams	0.315	1.34	3.2	0.483	-
	Fish shell	0.610	0.74	14.5	3.52	-
	Crab+shrimp	0.993	1.31	27.6	2.70	-
	Crab+prawn	1.25	0.64	32.4	2.31	138
Demineralized wastes	Crab	0.114	1.98	28.1	0.416	-
	Shrimp	0.652	0.40	33.48	3.42	-
	Prawn	0.445	1.61	35.70	0.516	-
	Squilla	0.521	0.83	29.10	0.215	-
	Squid	0.449	1.39	16.22	2.03	-
	Clams	0.505	0.76	12.64	4	-
	Crab+shrimp	0.522	1.15	**42.3**	**6.20**	105.55
	Crab+prawn	0.674	1.24	**39.5**	**4.27**	-

However, the demineralized crab + shrimp and crab + prawn powders gave the highest chitinase and protease activities 42.3 and 39.5 U/ml for the chitinases and 6.2 and 4.27 U/ml for the protease respectively. In addition, the deproteination folds also were high in the demineralized wastes than the crud one. It seemed that the demineralization (acid treatment) of the chitinous waste generally enhance the chitinase and protease production as reported by many workers[26], this may be due to the fact of the acid treatment eliminate the salts and partially hydrolyze the chitin render it accessible by the chitinases and the proteases also synergistically participated indirectly in the chitin degradation as demonstrated by Miyamoto *et al.* 2003[27], those found four serine proteases and three metalloproteases were involved in the chitinolytic system of the marine bacterium, *Alteromonas* sp. 0.7 and those enzymes were chitin inducible and increase the chitin hydrolysis efficiency by about 15%. On the other hand, we noticed in the Table some lower protease activities and lower deproteination folds, the possible reasons may be attributed to Maillard reaction associated with the cooking and high temperature drying process. Sikorski and Ruiter, 1994[28]. reported that the different drying conditions slightly decrease the nutritional properties of seafood proteins depending on the severity of heat treatment, oxidation and cross-linking between the amino acid residues. Maillard type non-enzymatic browning reactions may involve NH_2 groups in amino acid residues as well as in free amino acids; this reaction renders the protein in shrimp and crab shell powder (SCSP) resistant to protease

treatment [29]. In addition, the acid treatment sometimes did not yield favorable results possibly due to the destruction of many proteins that required for the protease induction and activity.

4. Conclusion

The microbial reclamation of crustaceous processing wastes such as crab and shrimp shells for bacterial growth and multiple enzymes production seems to be cost effective and promising approach, which can pave the way for the industrial production of valuable compounds via submerged fermentation (SMF).

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