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Full Length Research Paper

Production, purification and characterization of polysaccharide lytic enzymes of a marine isolate, *Bacillus cereus* NRC-20 and their application in biofilm removal

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In screening for marine bacterial polysaccharide lytic enzymes, the most potent isolate was identified as *Bacillus cereus* NRC-20. This strain showed high alginase, dextrinase, pectinase and carboxymethyle-cellulase productivity when production medium was adjusted at pH 6.0 and contains (g/l): 3.0 sodium alginate, 3.0 malt extract, 30.0 jatropha cake and inoculated with 8% (v/v) of a 24 h old cell suspension and incubated at 30°C for 48 h on a rotary shaker at 200 rpm. These enzymes mixture were precipitated by 60% acetone and purified by using sephadex G-100, whereas SDS-PAGE suggested a molecular weight of approximately 17, 20, 89 and 279 KDa, respectively. The optimum partially purified enzymes activity when enzyme protein concentration was 27.4 mg/ml and substrate concentration 5.0 g/l shown after 90 min of incubation at 30°C and pH 5 and stable at 60°C for 60 min. The enzymes activities were enhanced in the presence of 0.02 M Na^+ and K^+ ions and completely inactivated by Mg^{+2}, Cu^{+2}, Co^{+2} and Pb^{+2} The enzymes mixture affected the matrix integrity of different microbial biofilms artificially grown on stainless steel sheets of *Bacillus subtilis* NRRL B-4219, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27953 and *Escherichia coli* ATCC 25922.

Key words: *Bacillus cereus*, polysaccharide lytic enzymes production, fermentation parameters, enzyme characterization, biofilm removal.

INTRODUCTION

The structure of biofilm matrix is a mixture of micro-colonies with water channels in-between and an assortment of cells and extracellular polymers (polysaccharides, glycoproteins and proteins (Christensen, 1989;

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Abbreviations: DNSA, Di-nitrosalicylic acid; **EPS,** extracellular polysaccharides; **PPE,** partially purified enzymes; **CMC,** carboxymethyle cellulose.

Table 1. Different media composition involved in enzymes production.

Different media used	Composition (g/l sea water)
Med.1 (Hansen et al., 1984)	Yeast extract 5.0, sodium alginate 5.0, 0.6mM $MgCl_2$
Med.2 (Hansen et al., 1984).	Peptone 5, Meat extract 1.0, Yeast extract 2, Sodium chloride 5.0,sodium alginate 5.0
Med.3 (Alexeeva et al., 2002)	bacto-peptone 2.0, casein hydrolysate 2.0, yeast extract 2.0, glucose 1.0, K_2HPO_4 2.0, $MgSO_4$ 0.05, 50% sea water, 50% distilled water
Med.4 (Manyak et al., 2004).	Yeast extract 1.0, sodium alginate 2.0
Med.5 (Hansen and Nakamura, 1985).	Peptone 5.0, Meat extract 1.0, Yeast extract 2, Sodium chloride 5.0, sodium alginate 1.0, 30 mM $MnSO_4$
Med.6 (Oldak and Trafny, 2005; Jain and Ohman, 2005).	Tryptone 1.0, yeast extract 0.5, NaCl 0.5

Flemming et al., 1992; Costerton et al., 1995). Biofilms can cause many problems in industry such as, increased frictional resistance to fluids on water conduits and in ship hulls (fouling), decreased heat transfer from heat exchangers, corrosion of metallic substrata, contamination of foods in food industry (Johansen et al., 1997; Xiong and Liu, 2010).

Mechanical cleaning (scrubbing, sonication, freezing and thawing) of biofilms can be costly and biocides are ineffective due to the reduced susceptibility and acquired resistance of microorganisms in biofilms to antimicrobial agents (Walker et al., 2007). Also, excessive use of antimicrobials could be toxic and damaging to the environment (de Carvalho, 2007). The use of polysaccharide lytic enzymes for biofilm removal is one of the attractive strategies for biofilm problem elimination. Enzymes remove biofilms by destroying the physical integrity of the extracellular polysaccharides (EPS) matrix where a mixture of hydrolytic enzymes could be employed to degrade the biofilm matrix resulting in complete removal of biofilms and enhancement of the antimicrobial activity (Manyak et al., 2004; Xavier et al., 2005).

Numerous bacteria can produce polysaccharide lytic enzymes, but most are marine bacteria (Manyak et al., 2004). Marine bacteria of the genus *Bacillus* are known to produce both antimicrobial metabolites and enzymes (Ivanova et al., 1999; Peterson et al., 2006).

Microbial EPS lytic enzymes are a wide range of enzymes most of which are highly specific. These specific polysaccharide-degrading enzymes, when applied on a polysaccharide matrix can yield oligosaccharide fragments, which are amenable to NMR and other analytical techniques. Complex systems containing various mixtures of enzymes may also be effective in many applications such as biofilm removal from different surfaces preventing health problems (Chen and Stewart, 2000; Donlan and Costerton, 2002; Manyak et al., 2004; Xavier et al., 2005; Johanson et al., 2008; Häussler and Parsek, 2010).

The aim of this study was to obtain marine bacterial isolates from different localities in Egypt that have the maximum production and activity of polysaccharide lytic enzymes which are effective in the degradation and removal of bacterial biofilm that are most commonly found causing problems in many medical and industrial fields.

MATERIALS AND METHODS

Microorganisms and culture conditions

Twenty eight (28) bacterial isolates were obtained from different localities of marine sea water in Egypt (Ain-Sokhna-Hurghada-Sharm Elsheikh) and were tested for their productivity of extracellular polysaccharide lytic enzymes. The growth medium used was nutrient agar which is composed of (g/l): yeast extract 2.0, meat extract 1.0, peptone 5.0, NaCl 5.0 (0.8 M), agar 15.0 and supplemented with sodium alginate 5.0 and adjusted at pH 7. For the production of alginase enzyme, the following minimal medium (Manyak et al., 2004) was used (g/l sea water): yeast extract 1.0 and sodium alginate 2.0 and pH was adjusted to 7.0. 5 ml of the bacterial isolate suspension (72 h old focus 7×10^6 cfu/ml) was added to 250 ml shake flask containing 45 ml of the medium, the culture was incubated at 30°C for 48 h under shaking (200 rpm). The culture supernatant was collected by centrifugation at 6000 rpm for 10 min and is considered to be the crude polysaccharide lytic enzymes mixture. Supernatant was frozen until use and culture was run in duplicate.

Pseudomonas aeruginosa ATCC 27953, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* NRRL B-4219 as biofilm producing bacteria were obtained from American Type Culture Collection, Rockville, MD and Northern Regional Research Laboratory, Peoria, IL, United State. The strains were maintained in nutrient agar.

Optimization for maximum enzyme production

The different parameters were tested to obtain the maximum enzyme production including: different fermentation media (Table 1), different carbon source concentrations of the most potent fermentation medium and different nitrogen sources (yeast extract, casein hydrolysate, peptone, beef extract, urea, malt extract, tryptone, soy bean, N-acetylglucosamine, ammonium chloride, ammonium sulphate and/or sodium nitrate (at equimolecular basis to yeast extract present in the original medium) and different medium pH (4-9) was adjusted by acetate, phosphate and/or carbonate-bicarbonate buffers. Different agricultural waste material additives (rice shell, rice stem, molasses, jatropha cake, apple

peels, carrot peels and/or potato peels) were used as an inducing waste material. Finally the optimum medium was inoculated with different inoculums size. Also, incubation temperature (25-40°C), time of incubation (24-27 h) under different agitation speeds were evaluated.

Determination of polysaccharide lytic enzyme activity

All enzymatic activities in culture supernatant were determined at 30°C. All assays were run in triplicate, standard deviations of mean of subsamples was calculated by Microsoft excel program for all results obtained.

To assay the activity of polysaccharide lytic enzymes (alginase, dextrinase, pectinase and carboxymethyl cellulase (CMCase): 0.2 g of one of the following polysaccharides separately and respectively, sodium alginate, dextrin, pectin and CMC dissolved in 100 ml of 0.2 M phosphate buffer (pH 7) were used as substrates (Sumner and Sisler, 1944). The reaction mixtures was started by mixing 0.3 ml of enzyme source with 0.7 ml of substrate solution, and was stopped after 1 h of incubation, by adding 1 ml of dinitrosalicylic acid reagent (2.14% NaOH, 0.63% 3, 5-dinitrosalicylic acid, 0.5% phenol, all from Sigma) and immersion of the test tubes in a boiling water bath for 5 min. After cooling in tap water, absorption was measured at 575 nm. One unit enzyme was expressed as 1 µg/ml reducing sugar equivalent to D-glucose librated as an indication of enzyme activity per reaction time (Manyak et al., 2004).

Estimation of total protein

The total protein of the enzymes mixture was determined using the method of Lowry et al. (1951) with Bovine Serum Albumin (BSA) as a standard.

Partial purification and characterization of the lytic enzymes

Fractional precipitation with acetone was carried out by slowly adding with stirring a certain volume of cold acetone to 1 L of the culture filtrate (kept in an ice bath) until the required concentration of acetone was reached. Then after removing the precipitated fraction by centrifugation at 10.000 rpm for 15 min in a cooling centrifuge at 4°C, further acetone was added to the remaining supernatant to reach the next desired concentration and the process was repeated until the final concentration of the precipitant reached 100% (v/v). Several enzyme fractions were thus obtained at the corresponding concentration. The precipitate was dissolved in 1 ml of phosphate buffered solution. Enzyme activity and protein content of the concentrates were determined.

To examine the influence of several factors affecting their activities, the partially purified enzyme at different protein concentrations (13.7-48 mg/ml) were incubated each with the different specific substrates separately at different incubation temperatures ranging from 25-40°C for different time intervals (30-150 min) and at different degrees of pH (4-9) by using different buffer systems(0.2 M) such as acetate buffer (pH 4.0 and/or 5.0), phosphate buffer (pH 6.0, 7.0 and/or 8.0) and carbonate-bicarbonate buffer (pH 9.0). The enzymes mixture was added at different protein concentrations (13.7-48 mg/ml) to the respective substrates, evaluating the effect of enzymes protein concentration on enzymes activities.

The effect of substrate concentration on enzymes activities was tested whereas, the enzymes mixture was added to each of the specific substrates separately (sodium alginate, dextrin, pectin and/or CMC), in concentrations ranging from 2.0 to 10.0 g/l. Also, the effect of addition of metal ions: Na^+, K^+, Mg^{++}, Cu^{++}, Co^{++} and Pb^{++} as chloride salts (0.02 M) on enzymes activities were tested.

The molecular weights of the different polysaccharide lytic enzymes (after fractionation of enzymes on sephadex column G-100) were determined by native SDS-PAGE using standard bovine serum albumin protein marker (18-116 KDa), protein contents were adjusted to 2 mg/ml per sample.

Biofilm formation and removal

Pseudomonas aeruginose ATCC 27953, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *B. subtilis* NRRL B-4219 were grown according to de Queiroz and Day (2007), a standardized suspension of *P. aeruginosa* (20×10^4 cfu/ml) and *E. coli*, *S. aureus* and *B. subtilis* (30×10^4 cfu/ml) was inoculated into flasks, containing 100 ml sterile trypton soy broth (TSB). Standard biofilms were obtained by attachment of bacterial cells on sterile stainless steel sheets (ALSI 304) (1 × 1 cm) that are used in the hospitals, clamped vertically to a Teflon carousel, which was placed inside the flasks.

The flasks were incubated at 30°C for six days with continuous agitation at 200 rpm. During the biofilm growth, about 25 ml of the inoculating medium was replaced and fed with an equal volume of sterile TSB every 48 h to keep the bacterial cells at the exponential phase. At 6 days, bacterial suspensions containing the planktonic cells were discarded from the flasks. The sheets were withdrawn and dipped into saline solution (0.9%, w/v) to discard loosely attached cell, before being used as standard biofilm samples in removal tests.

To assay the biofilm removal efficiency, an standardized *P. aeruginosa* and *E. coli, S. aureus* and/or *B. subtilis* biofilm sheets were incubated into a falcon tube with 10 ml of the partially purified enzymes mixture at 30°C for 30 min without agitation, the biofilms with no enzymes were used as control. After incubation period, the sheets were then soaked for 1 min in saline solution (0.9%, w/v) and the effect of enzymatic activity on the biofilm was evaluated using the scanning electron microscope.

Samples preparation for scanning electron microscopy

Stainless steel sheet samples were fixed with 6% glutaraldehyde in 0.2 mol l^{-1} cacodylate buffer, then samples were dehydrated in ethanol. After the drying step, samples were critically dried with CO_2 (Molobela et al., 2010). Samples were coated with gold/palladium in an Edwards S-150 sputter coater and visualized using a scanning electron microscope (Quanta EFG 250).

RESULTS

In this work, some local marine bacterial strains were tested for their productivity of extracellular polysaccharide lytic enzymes. From the positive strains, the bacterial isolate NRC-20 was selected for further studies whereas it grew well and produce high level of alginase activity (23.1 µg/ml), and this strain was identified as *B. cereus* NRC-20.

Interaction between production of polysaccharide lytic enzymes and environment

Different fermentation media were used for polysaccharide lytic enzymes production (Table 1) and results

Table 2. Effect of different fermentation media on the polysaccharide lytic enzymes production by *Bacillus cereus*NRC-20.

Medium no.	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
1 (Hansen et al.,1984)	0.18±0.10	33.3±0.03	23.9±0.1	164.4±0.04	59.0±0.06
2*(Hansen et al., 1984)	0.13±0.15	23.9±0.07	16.9±0.05	148.0±0.06	47.4±0.13
3 (Alexeeva et al.,2002)	0.37±0.18	30.9±0.12	25.8±0.05	170.9±0.03	55.6±0.06
4 (Manyak et al., 2004) (minimal medium)	0.18±0.09	35.5±0.08	30.7±0.07	172.6±0.10	71.8±0.09
5 (Hansen and Nakamura, 1985)	0.40±0.06	27.3±0.14	17.6±0.13	162.8±0.09	52.2±0.07
6 (Jain and Ohman,2005)	0.17±0.04	27.2±0.09	23.2±0.09	98.6±0.05	55.4±0.14

*Control

Table 3. Effect of different concentrations of sodium alginate on the polysaccharide lytic enzymes production medium by *B. cereus* NRC-20.

Sodium alginate (g%)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
0.1	0.16±0.07	22.8±0.15	18.2±0.09	104.8±1.05	46.8±0.04
*0.2	0.18±0.09	35.9±1.30	30.7±0.06	172.9±1.08	71.6±0.09
0.3	0.25±0.04	39.9±0.70	45.8±0.19	197.2±0.05	75.6±1.50
0.4	0.33±0.10	32.1±1.07	43.2±0.57	334.3±0.12	19.2±1.04
0.5	0.38±0.08	27.8±2.05	30.7±1.03	112.4±2.08	17.5±1.10
0.6	0.35±0.15	20.2±01.45	13.4±02.06	67.8±0.09	13.8±0.09

*Control.

indicated that, minimal medium no.4 containing only sodium alginate as a carbon source, yeast extract and sea water exhibited the highest potential for the production of alginase, dextrinase, pectinase and CMCase (35.5, 30.7, 172.6 and 71.8 µg/ml), respectively (Table 2).

The addition of the sodium alginate at concentration of 0.3% (w/v), as a sole carbon source to the fermentation medium resulted in a maximum production of alginase, dextrinase, pectinase and CMCase (39.9, 45.8, 197.2, 75.6 µg/ml) respectively. Increasing the concentration of sodium alginate up to 0.4 g % resulted in decreasing the enzymes production, while the amount of pectinase increased reaching 334.3 µg/ml (Table 3).

Results presented in Table 4 reveal that, organic nitrogen sources yielded better enzymes production than inorganic nitrogen sources. Malt extract (1g %) is the best nitrogen source used, producing values 78.1, 92.5, 282.1, 98 µg/ml for the activity of alginase, dextrinase, pectinase and CMCase, respectively.

The effect of addition of seven different industrial and agro-waste materials as an inducer agents on polysaccharide lytic enzymes production by *B. cereus* NRC-20 were studied using seven different agriculture and industrial waste materials rich in polysaccharides. The results indicated that the maximum enzymes production was obtained in the presence of jatropha cake (Table 5). Thus, different concentrations of jatropha cake (10-50 g/l) were used to evaluate the best concentration, maximum enzymes yield for the production of polysac-charide lytic enzymes were obtained at concentration of 30 g/l (Table 6).

Results from Tables 7, 8, 9 and 10 showed that the environmental condition for the maximum enzymes production was obtained when the fermentation medium was adjusted at pH 6.0 and inoculated with 8% (V/V) of the bacterial cell suspension and incubated at 30°C and 200 rpm for 48 h. The enzyme production was 83.6, 124.6, 292.6, 97.5 µg/ml for alginase, dextrinase, pectinase and CMCase, respectively (Table 11).

Partial purification of enzymes and its properties

The enzymes mixture was precipitate by using acetone 60%. For partially purified enzyme (PPE) assay conditions, increasing the reaction temperature up to 35°C increased the PPE activities (209.2, 229.4, 339.7 and 220.3 µg/ml) for alginase, dextrinase, pectinase and CMCase, respectively (Table 12).

Incubation time of PPE with their respective substrates influences the enzymes activities to a great extent as demonstrated in Table 13. Whereas, each enzyme activity of the PPE mixture reached its maximum after

Table 4. Effect of different nitrogen sources on polysaccharide lytic enzymes production medium by *Bacillus cereus* NRC-20.

Nitrogen source	Enzymes activity (U/ml)			
	Alginase	Dextrinase	Pectinase	CMCase
*Yeast extract	39.9±0.07	45.2±0.06	199.1±0.12	75.62±0.04
Peptone	35.0±0.07	65.7±0.03	279.2±0.09	112.3±0.07
Casein hydrolysate	20.3±0.03	21.2±0.08	59.8±0.03	28.7±0.12
Beef extract	40.6±0.06	84.8±0.05	310.2±0.06	43.1±0.09
Urea	24.6±0.08	19.2±0.08	62.5±0.04	18.4±0.07
Malt extract	78.1±0.04	92.5±0.14	282.1±0.05	98±0.05
Tryptone	36.8±0.09	91.2±0.09	266.3±0.08	75.6±0.13
Soy bean	55.0±0.04	79.3±0.07	151.2±0.02	40.3±0.09
N.acetylglucoseamine	18.4±0.05	21.2±0.06	33.6±0.05	17.9±0.04
Ammonium Sulphate.	23.4±0.03	26.4±0.03	64.4±0.02	26.1±0.05
Ammonium Chloride	27.1±0.12	21.9±0.10	49.7±0.04	30.4±0.08
Sodium Nitrate	22.8±0.10	23.1±0.08	57.7±0.06	26.5±0.07

*Control.

Table 5. Effect of addition of different waste materials on the production medium of polysaccharide lytic enzymes by *Bacillus cereu*s NRC-20.

Waste material (20 g/l)	Enzymes activity (U/ml)			
	Alginase	Dextrinase	Pectinase	CMCase
Rice shell	26.6±0.07	42.5±0.04	150±0.06	20.9±0.09
Rice stem	82.8±0.09	64.1±0.09	112.0±0.08	55.3±0.07
Jatropha cake	120.0±0.03	138.8±1.3	292.0±0.09	95.5±0.08
Molasses	22.8±1.00	23.4±0.08	168.0±1.10	33.7±0.05
Carrot peels	50.6±0.05	63.6±1.2	149.68±0.06	29.68±0.08
Apple peels	47.5±0.05	60.1±0.09	147.18±1.60	33.75±1.10
Potato peels	47.8±0.08	68.7±0.07	153.75±0.09	30.63±1.40
*control	83.2±0.07	124.9±0.09	292.4±0.10	92.4±0.09

*Control.

Table 6. Effect of different concentrations of jatropha cake on the production medium of polysaccharide lytic enzymes by *Bacillus cereus* NRC-20.

Jatropha cake concentration (g/L)	Enzymes activity (U/ml)			
	Alginase	Dextrinase	Pectinase	CMCase
10	69.6±0.09	43.4±0.07	286.9±0.07	36.9±1.2
*20	120±0.05	140.2±1.6	292.7±1.04	98±0.09
30	255±0.02	118.9±0.06	392.2±0.09	61.2±0.04
40	202.5±0.09	120.7±0.08	250.8±0.05	54.8±0.05
50	176.2±0.08	93.6±0.03	216.4±0.04	49.7±0.09

*Control.

90 min of incubation giving the values of 227.1, 237.4, 384.6 and 217.7(μg/ml) for alginase, dextrinase, pectinase and CMCase, respectively and the highest activity of enzymes mixture has been achieved at pH5. Data in Table 14 reveals that the enhancement of the lytic enzymes activities was proportional to its concentration up to 27.4 mg/ml, whereas the PPE activities were 250.3, 249.1 and 648.1 μg/ml for alginase, dextrinase

Table 7. Effect of different incubation temperatures on the polysaccharide lytic enzymes production by *Bacillus cereus* NRC-20.

Temperature (ºC)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
25	0.29±0.09	59.7±6.25	72.6±0.49	150.4±14.93	43.8±3.03
*30	0.38±0.05	78.1±3.09	92.3±9.13	282.8±6.55	98.5±12.12
35	0.37±0.12	69.3±1.42	89.1±2.07	234.3±2.39	79.5±2.09
40	0.32±0.07	32.1±2.97	50.4±0.75	134.5±17.06	23.8±1.07

*Control.

Table 8. Effect of different incubation periods on the polysaccharide lytic enzymes production by *Bacillus cereus* NRC-20.

Incubation period (h)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
24	0.10±0.06	55.7±3.05	50.2±1.02	151.2±10.05	46.3±8.08
*48	0.38±0.05	78.8±5.67	92.7±7.06	282.8±16.47	98.6±12.10
72	0.24±0.08	35.9±1.10	66.8±3.03	156.2±18.04	38.2±6.19

*Control.

Table 9. Effect of different pH values on the polysaccharide lytic enzymes production by *B. cereus* NRC-20.

pH value	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
4	0.30±0.05	21.2±0.64	29.4±3.33	239.4±17.07	38.2±4.14
5	0.34±0.09	56.2±2.83	39.2±4.10	265.1±9.05	68.4±4.08
6	0.35±0.08	81.3±6.08	98.2±1.77	299.8±24.09	98.9±3.08
***7**	0.38±0.03	78.8±5.06	92.7.8±8.11	292.7±14.03	98.3±0.53
8	0.37±0.10	55.0±5.09	45.9±3.05	150.4±8.06	34±1.11

*Control.

Table 10. Effect of different inoculum sizes on the polysaccharide lytic enzymes production by *Bacillus cereus* NRC-20.

Inoculum size (ml %)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
4	0.27±0.08	25.0±0.09	52.1±0.05	187.4±0.04	27.4±0.02
6	0.33±0.12	31.1±0.02	116.5±0.12	268.7±0.07	43.6±0.05
8	0.34±0.10	83.6±0.03	124.6±0.08	299.6±0.09	98.8±0.04
*10	0.35±0.09	81.0±0.07	98.1±0.06	299.3±0.07	98.9±0.08

*Control.

and pectinase, respectively, while CMCase had the highest activity (316.2 μg/ml) at protein concentration of 48 mg/ml (Table 15). On the other hand, by increasing the substrate concentration up to 5 g/l enhanced the activities of the different enzymes giving the values 247.8, 250.1, 422.7 and 239.2 μg/ml for alginase, dextrinase, pectinase and CMCase, respectively, while the further increase in substrate concentrations resulted

Table 11. Effect of different agitation speeds on the polysaccharide lytic enzymes production by *Bacillus cereus* NRC-20.

Agitation speed (rpm)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
0	0.27±0.06	30.0±5.6	30.0±2.1	200.53±0.9	65±12.4
150	0.33±0.08	59.3±0.09	80.5±0.05	235.1±0.05	67.4±2.06
*200	0.34±0.04	83.2±0.07	124.9±0.69	298.4±0.10	98.6±7.09
250	0.38±0.09	64.5±0.03	62.3±1.9	189.2±0.13	45.7±0.08

*Control.

Table 12. Effect of different incubation temperatures on the relative activity of partially purified enzyme.

Temperature (°C)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
25	70.4	81.8	88.8	76.8
*30	100.0	100.0	100.0	100.0
35	105.4	124.3	113.7	144.5
40	52.3	72.9	67.3	61.5

*Control.

Table 13. Effect of different assay incubation times on the relative activity of partially purified enzyme.

Time (min)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
30	85.5	69.8	68.7	72.1
*60	100.0	100.0	100.0	100.0
90	112.1	110.0	125.1	114.3
120	93.4	94.9	124.2	106.0
150	76.3	54.2	82.8	98.6

*Control.

Table 14. Effect of different assay pH values on the relative activity of partially purified enzyme.

Buffers	pH	Relative activity (%)			
		Alginase	Dextrinase	Pectinase	cmcase
Acetate	4	103.8	102.9	109.1	46.5
	5	126.0	115.2	119.7	105.4
	*6	100.0	100.0	100.0	100.0
Phosphate	7	89.7	91.6	89.4	84.2
	8	88.3	84.7	79.8	84.1
Carbonate- bicarbonate	9	83.4	74.8	72.7	79.1

*Control.

in the decrease in enzyme activity (Table 16). Among different added metal ions, K^+ was a strong activator of the enzymes mixture activities, Na^+ was a moderate activator while, Cu^{++}, Co^{++}, Pb^{++} are considered as enzyme

Table 15. Effect of different enzymes protein concentrations on the relative activity of partially purified enzyme.

Enzyme concentration (mg/ml)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
0.1	78.1	83.6	93.6	86.3
0.2	91.2	96.8	98.7	94.8
*0.3	100.0	100	100.0	100
0.4	85.5	94.9	94.9	103.1
0.5	74.2	82.1	87.3	125.4
0.6	67.8	81.5	62.2	125.3

*Control.

Table 16. Effect of different substrates concentrations on the relative activity of partially purified enzyme.

Substrate concentration (g %)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
*0.2	100.0	100.0	100.0	100.0
0.5	122.0	118.7	128.1	136.2
0.8	86.1	91.2	86.1	91.6
1.1	71.3	76.6	56.8	71.0

*Control.

Table 17. Effect of addition of different metal ions on the relative activity of partially purified enzyme.

Metal ions (0.02 M)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
Na^{+}	138.1	156.0	171.5	136.6
K^{+}	110.0	144.1	193.5	92.9
Mg^{++}	100.2	66.6	108.8	77.1
Cu^{++}	61.4	67.2	50.1	80.8
CO^{++}	70.4	60.9	58.4	78.3
Pb^{++}	59.1	59.7	64.0	70.5
*control	100.0	100.0	100.0	100.0

*Control.

inhibitors (Table 17).

Thermal exposure of the PPE to different temperatures for different time intervals (not tabulated) demonstrated that, the enzyme mixture alginase, dextrinase, pectinase and CMCase are stable with increasing the reaction temperature, whereas the pectinase enzyme retained only 82.7% and dextrinase enzyme retained 98.1% of the relative activity after being exposed to 60°C for 120 min, while alginase and dextrinase enzymes started losing their activities after exposure to 70°C for 60 min, retaining 99.3, 96.3% relative activities, respectively. The molecular weights determined in the PPESDS-PAGE with Coomassie-brilliant blue staining mixture were: 17, 89, 279 KDa, respectively (Figure 1).

Effect of partially purified enzymes on biofilm removal

The microscopic studies of the effect of PPE mixture on *P. aeruginosa*, *E. coli*, *S. aureus* and *B. subtilis* biofilms revealed that the biofilms treated showed a reduction in the biofilm cells and substantial degradation of the extracellular polymeric substances (EPS) protecting the

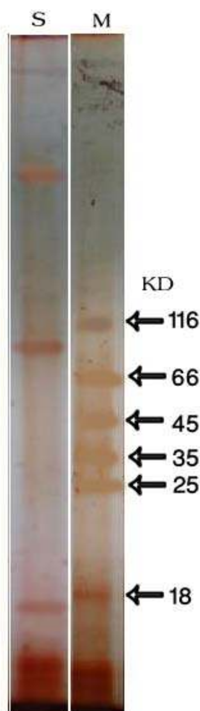

Figure 1. Molecular weight of partially purified enzymes of B. cereus. NRC-20 M: Protein marker (18-116 KDa); S: Bands of PPE.

pathogenic bacteria. The enzymes mixture was more effective in degrading EPS and biofilm removal of *S. aures* followed by *E. coli*, *P. aeruginosa* and then *B. subtilis* (Figures 2, 3, 4 and 5)

DISCUSSION

In response to environmental and health concerns on the extended use of antibiotics, it seems inevitable that greater reliance will be placed on alternative biological control techniques including the use of natural microbial derived substances like microbial enzymes. Marine derived microbial enzymes are used for many purposes including both medical and industrial fields, one of the most interesting uses of marine microbial enzymes is the use of enzymes for biofilm removal.

The application of enzymes for control of protein biofilm on surfaces and in closed pipelines is well known (Aldridge et al., 1994), while the application of enzymes to degrade EPS is promising and attractive in medical sciences and many industries where complete biofilm removal is essential. It is interesting in this study to focus on the removal of polysaccharide components of the extracellular polymeric substance (EPS) of *P. aeruginosa* ATCC 27953, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *B. subtilis* NRRL B-4219 biofilms as well studied bacterial organisms for biofilm formation using enzymes mixture isolated from marine *B. cereus* NRC-20.

Exopolysaccharides are a major component of most biofilm matrices (Orgaz et al., 2006; Fernández et al., 2012), the absence of exopolysaccharide synthesis and export, bacteria can adhere to the surfaces but unable to form multilayer biofilms and due to the heterogeneity of the extracellular polysaccharides within the biofilms, a mixture of exopolysaccharide lytic enzymes is necessary for sufficient degradation of bacterial biofilm (Xavier et al., 2005; Kaplan, 2010). Therefore, the investigated enzymes production and properties in this study are, a mixture of alginase, dextrinase, pectinase and carboxy-methyl cellulase produced by marine *B. cereus* NRC-20, whereas some investigations reported more and more microorganisms from marine habitats that can produce polysaccharide lyric enzymes (Kaplan, 2010) and they have been attracting more attention as a resource for new enzymes, because the microbial enzymes are relatively more stable and active than the corresponding enzymes derived from plant or animal (Gupta et al., 2003; Byrd et al., 2011).

The screening medium containing alginate as the only carbon source supported growth and best production of the multiple specificity enzyme mixture, this agrees with the findings of Manyak et al. (2004) who reported that, marine-derived microorganisms are useful sources of polysaccharide lyases, since they evolved powerful enzyme systems to take advantage of the ubiquitous marine nature. The best alginase producing marine bacterial isolate was identified using API 50CHB system and was found to be *B. cereus.* Heterotrophic *Bacillus* strains are considered to be species of a variety of habitats. Species of *Bacillus marinus, Bacillus badius Bacillus subtilis, B. cereus, Bacillus licheniformis, Bacillus firmus* and *Bacillus lentus* are often isolated from marine habitats as reported by Ivanova et al. (1999). The capacity of the *Bacillus* sp. to produce and secrete large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers (Namasivayam et al., 2011).

The production conditions of the lytic enzymes responsible for the degradation of the most common biofilm matrix polysaccharides have been investigated. Commercially available enzyme preparations have seldom proved the capability of degrading microbial heteropolysaccharides (Sutherland, 1999; Loiselle and Anderson, 2003; Chaignon et al., 2007).

The best fermentation medium for the different polysaccharide lytic enzymes production was the minimal medium containing only sodium alginate as a carbon source along with yeast extract and sea water. On the other hand, the rest of the enzymes production media containing other mineral supplements ($MnSO_4$ and

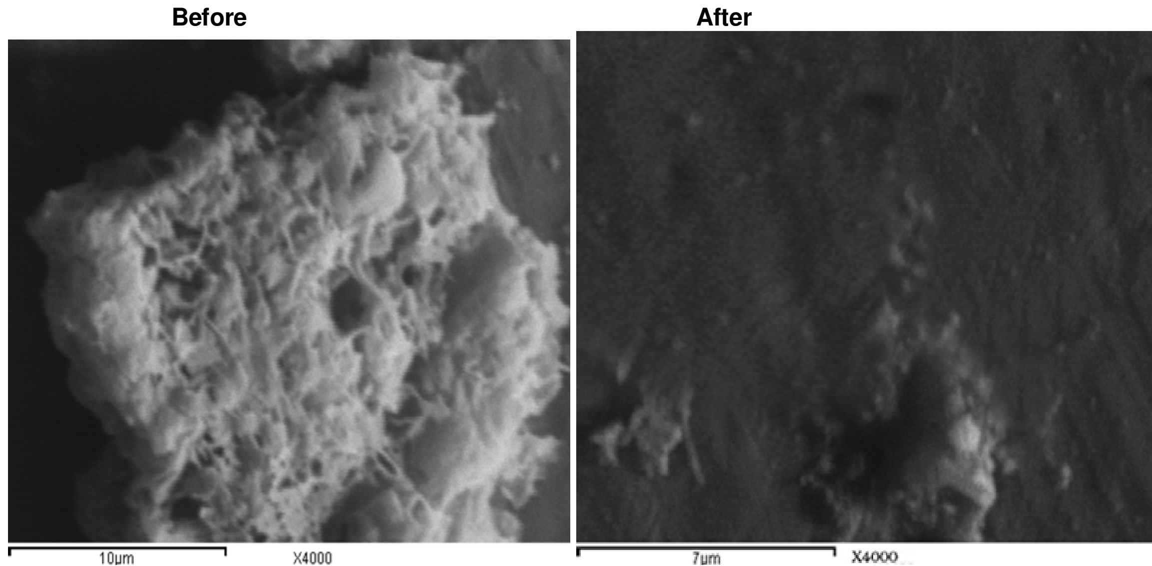

Figure 2. Six days old *P. aeruginosa* biofilm on stainless steel before and after partially purified enzyme treatment.

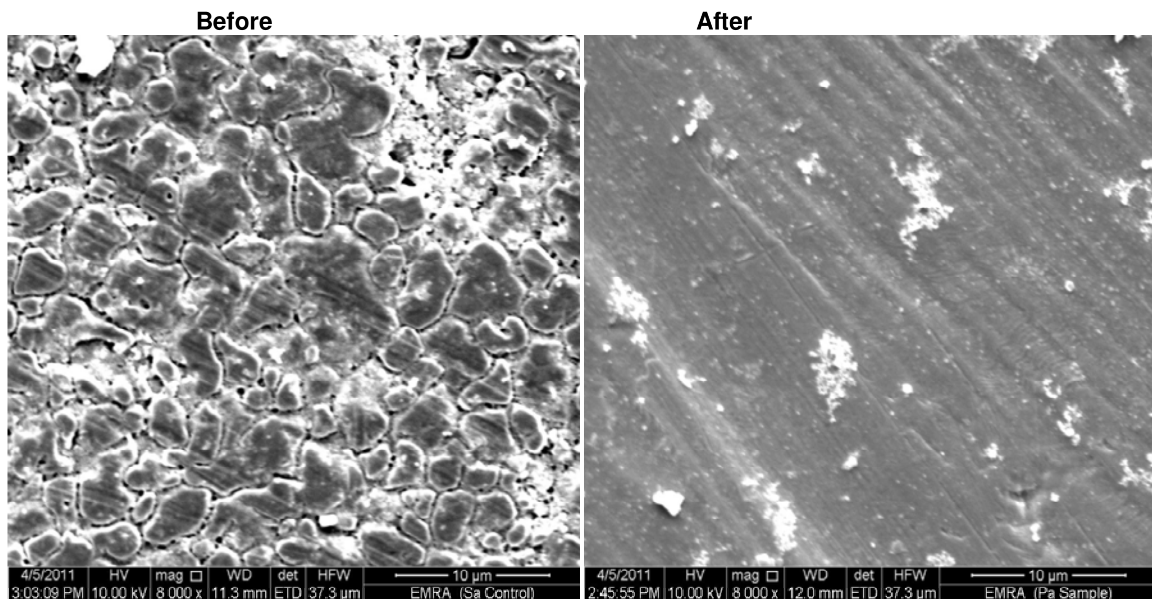

Figure 3. Six days old biofilm of *S. aureus* on stainless steel sheets before and after partially purified enzyme treatment.

$MgCl_2$) and other rich nitrogen sources (peptone, tryptone, casein hydrolysate) seemed to minimize the enzymes production. The best concentration of sodium alginate is 0.3 g%, best nitrogen source is malt extract 0.3 g%, provided the organic nitrogen sources yielded better polysaccharide lytic enzymes productivity than inorganic sources. This is in agreement with the results reported by Stoudt and Nollstadt (1982) and Namasivayam et al. (2011), indicating that the use of organic nitrogen sources such as yeast extract, corn steep liquor are better than inorganic ingredients together with the addition of the trace substances.

Bacillus cereus NRC-20 was found to give maximum enzyme production at 30°C.This agrees with the results by Namasivayam et al. (2011) that, most *Bacillus* sp. need 32-37°C for pectinase enzyme production. Optimal pH for enzyme production in this study was found to be pH 6.0. This agrees with the reported data of Liu and Chi-Li (1988), indicating that the best alginase production is at pH 5-6.5, using sodium alginate as the carbon source.

The best inoculum size was found to be 8% (v/v), incubated for 48 h on a rotary shaker adjusted at 200

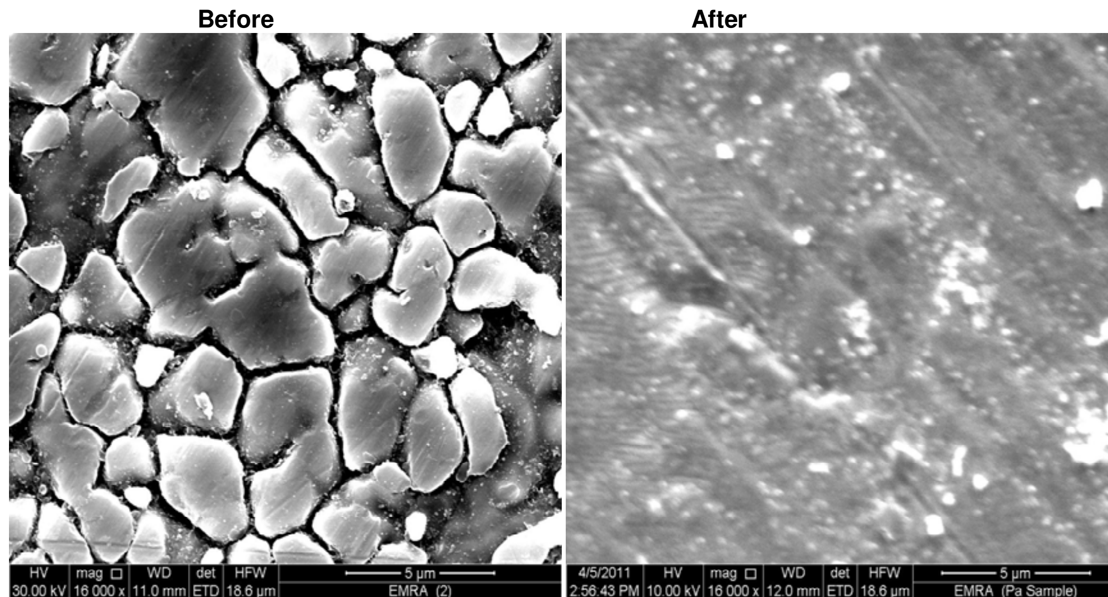

Figure 4. Six days old biofilm of *E.coli* on stainless steel sheets before and after partially purified enzyme treatment

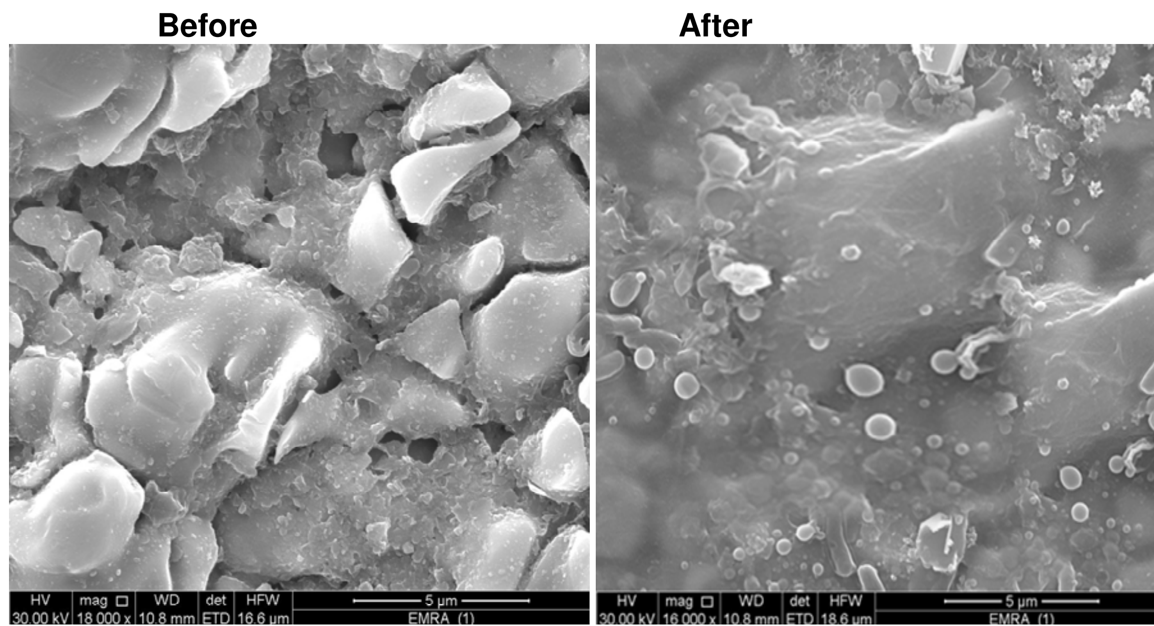

Figure 5. Six days old *Bacillus subtilis* biofilm on stainless steel sheets before and after partially purified enzyme treatment.

rpm. This is in agreement with reported data by Manyak et al.(2004) who harvested his polysaccharide lytic enzyme mixture after 48 h.

Several tested waste material additives were tested as inducer agents for the production of polysaccharide lytic enzymes. Jatropha cake which is the wastes of vegetable oil seed extraction produced promising amounts of polysaccharide lytic enzymes since, the plant seed will contain a number of lipoproteins and lipopolysaccharides like amylose, glucan and pectin which will increase the productivity of polysaccharide lytic enzymes (Achten et al., 2008). On the other hand, wheat bran acts as a better agro substrate and magnesium chloride was supplement for better production of pectinase

(Namasivayam et al., 2011).

Precipitation of the PPE from the supernatant of the enzyme production medium by *Bacillus c.*-NRC-20 was carried out by 60% acetone, which resulted in the highest yield of enzymes activities. The previous results are in agreement with the results reported by Stoudt and Nollstadt (1982), they found that the enzymes hydrolyzing the plaque matrix were precipitated by 60% acetone. On the other hand, pectinase from *B. cereus* isolated from solid waste was precipitated by 80% ammonium sulphate (Lopez et al., 2010; Namasivayam et al., 2011); also, the polysaccharide lytic enzymes mixture, reported by Manyak et al. (2004), was precipitated by 80% ammonium sulphate.

The PPE had the best activity when 27.4 mg/ml of the enzymes mixture protein was incubated with 5.0 g/l of the corresponding polysaccharide substrates at 35°C for 90 min at pH 5. Wang et al. (2004) reported that in an enzyme mixture from *Penicillium roquefort*, the dextrinase enzyme activity was stable in a pH range of 5.0 to 5.3 and when SDS-PAGE was applied, three enzyme protein bands appeared (17, 89, 279 KDa) while, a bifunctional alginate lyase was separated and polyacrylamide gel electrophoresis separation resulted in two alginases having molecular weights of 23.0 and 33.9 KDa.

Many antimicrobial agents fail to penetrate the biofilm of bacterial pathogens due to EPS which acts as barrier protecting the bacterial cells within. The alternative will be the use of compounds which can degrade the EPS of the biofilm (Loiselle et al., 2003; Walker et al., 2007). Enzymes have been proven to be effective for the EPS degradation of the biofilms (Lequette et al., 2010) and remove biofilms directly by destroying the physical integrity of the EPS by weakening the carbohydrate, proteins and lipid components making up the structures of the EPS through the degradation process (Xavier el al., 2005).

Interestingly, the electron microscope photos of stainless steel sheets of biofilms under test were examined before and after PPE mixture treatment and the enzymes mixture under test was effective for the removal of the different biofilms, showing almost complete removal of *P. aeruginoa, S. aureus* and *E. coli* biofilms. The major components of the polysaccharide matrices for these biofilms are alginate for *P. aeruginosa* (May et al., 1991), cellulose for both *S. aureus* and *E. coli* (da Re and Ghigo, 2006; Seidl et al., 2008). On the other hand, the lytic enzymes mixture from *B. cereus* NRC-20, showed little effect on the biofilm of *B. subtilis,* since the major component of this biofilm matrix is γ-glutamate (Morikawa et al., 2006), and the enzyme mixture does not contain a lytic enzyme for this polysaccharide

Conclusion

The present study clearly indicates that the *B. cereus* NRC-20 can be used for the production of polysaccharide lytic enzymes mixture (alginase, dextrinase, pectinase and carboxymethyle cellulase) used for biofilm removal of a number of pathogenic bacteria including: *P. aeruginosa, S. aureus, E. coli* and to a lesser extent *B. subtilis*. These enzymes represent a method to eliminate biofilms through naturally derived microbial products, minimizing the possible hazards of large quantities and extended use of antibiotics which can be damaging and not effective in many cases. The constant periodical use of these polysaccharide lytic enzymes is recommended for eliminating existing biofilms and preventing the formation of new ones.

Conflict of interest

The authors declare that they have no conflict of interest.

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