Purification, Characterization and Immobilization of Alginase Produced by *Bacillus* sp Associated with *Sargassum wightii*

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Abstract Seaweed sample was collected from tide pools and rock surfaces in the Kanyakumari coastal region. The bacterial isolate was identified based on the morphological and cultural characteristics. The effect of different pH on alginase production by *Bacillus* sp. is at pH 8.0 (10.9 ± 0.34 IU/ml). Among the various temperatures tested, maximum alginase production was recorded in 40°C (15.9 ± 0.39 IU/ml). The effect of metal ions tested at calcium chloride (32.41 ± 0.47 IU/ml). Sodium alginate matrix was increased gradually up to 24 hrs (0.53 ± 0.012 IU/ml). Agar-Agar matrix was better steadily up to 24 hrs (0.6 ± 0.017 IU/ml). Gelatin matrix was increased gradually up to 24 hrs (0.37 ± 0.10 IU/ml). There are four variables used for the factorial analysis including pH, temperature, alginate and ammonium chloride namely X1, X2, X3 and X4 respectively. There was a considerable variation in the alginase production depending on the four chosen variables. The maximum alginase production of 36.4 IU/ml was achieved by RSM (CCD).

Keywords Alginase, Immobilization Matrix, RSM

1. Introduction

Alginate is such an important component of the outer surface in the context of chronic *P. aeruginosa* lung infections in Cystic fibrosis. The researchers that first identified alginate on the surface of *P. aeruginosa* recognized enzymes that degrade this polysaccharide [1]. Cystic fibrosis (CF) is the most common fatal genetic disease among the Caucasian population producing a multisystem disorder. CF is characterized chiefly by chronic airways obstruction and infection and by exocrine pancreatic insufficiency with its effects on gastrointestinal function, nutrition, growth, maturation and reproduction [2]. This disease is the most common life-threatening genetic trait in Caucasian populations of European ancestry [3].

The alginic acid salt form alginate can be degraded by radiation or thermal treatment [4]. Biological degradation,

on the other hand, is generally conducted by an alginate lyase (EC 4.2.2.3) that acts on the 4-O-linked glycosidic linkage of alginate [5]. Alginate lyases have been isolated from a wide range of organisms, including algae, marine invertebrates, and marine and terrestrial microorganisms [6]. Most alginate degrading bacteria were originally found to be closely associated with marine algae or mollusks. Alginate lyases, also known as alginases or alginate depolymerases, catalyze the degradation of alginate by a $[\beta]$ elimination mechanism. Although all lyases perform essentially the same depolymerization action on alginate, each enzyme is defined by its individual characteristics and its preference for the glycolytic bond connecting M and G monomers. Although most organisms produce a single alginate lyase with defined substrate specificity, some marine animals and bacterial strains produce two or more enzymes [6].

Alginate can restrict the diffusion of certain antibiotics into the cell and can also inhibit several of the host's major antibacterial defense mechanisms, making it very difficult for CF patients to clear mucoid *P. aeruginosa* from their lungs [7]. Alginate lyases have been identified in a variety of bacteria, including marine bacteria [8].

Alginate oligosaccharides are depolymerization products of alginate by alginate lyases or physicochemical method. They have attracted considerable attention because of their applications in the food industry as growth promoters for plants and as therapeutic agents i.e., anticoagulants, tumor inhibitors and suppression of IgE [9, 10]. The alginate lyase are still being developed with poor sources, narrow substrate specificity and low enzyme activity and limit the generation of novel alginate oligosaccharides. The present study reveals that the selected isolates shows high enzyme activity and wide substrate specificity and also expand the potential applications of alginate lyases.

2. Materials and Methods

2.1. Collection and Processing of the Sample

Seaweed *Sargassum wightii* sample was collected from

tide pools and rock surfaces in the Kanyakumari coastal region. *Sargassum wightii* sample was collected using sterile scalpel and transferred to sterile bottle. Then the sample was used for further microbiological analysis.

2.2. Screening Alginase Producing Bacteria

Screening plates with a medium composed of 0.5 g NaCl₂, 1 g (NH₄)₂ SO₄, 0.5 g KH₂ PO₄, 1.5 g K₂HPO₄, 0.2 g MgSO₄, and sodium alginate 10g. The plates were then incubated at 37°C for 24hrs to form detectable colonies [11].

2.3. Alginase Assay

The culture was centrifuged at 10000 rpm for 10 mins at 4°C. The supernatant was used as crude enzyme source for alginase assay. A solution of 0.6 g sodium alginate was dissolved in phosphate buffer was used as the substrate. The reaction was performed by adding 1ml substrate to 1ml enzyme. Incubation was performed in a water bath at 37°C for 20 mins. The reaction was terminated by addition of 1ml of DNS reagent and tubes were kept at boiling water bath for 10 mins. After cooling the tubes at room temperature, 1ml of distilled water was added in each tube. The intensity of the colour was read at 540nm in UV-Vis spectrophotometer (Systronics, 119). Standard curve was performed with glucose solution. One unit of enzyme activity was defined as the amount of enzyme required for under assay condition. Enzyme activity was expressed in International units [11]. Alginase activity was calculated using this formula:

$$\text{IU/ml} = \text{concentration of glucose} / 1 \times 30 \times 0.180$$

2.4. Acetone Precipitation

The protein precipitation, double the amount of acetone was added to the culture supernatant solution and the solution was left overnight at 4°C, the supernatant was removed and pellet of precipitated protein was kept and dried at laboratory temperature [12].

2.5. Effect of pH

The effect of pH on alginase activity and stability were determined individually for crude alginase obtained from *Bacillus* sp. [10].

2.6. Effect of Temperature

The effect of temperature on the stability of alginase was determined in the temperature at pH 9 for 30, 60, 90, 120, 150 and 180 mins [10].

2.7. Effect of Metal Ions

The effect of various metal ions on alginase activity and stability were determined individually for crude alginase of *Bacillus* sp. [13].

2.8. Effect of Various Surfactants on Alginase Activity and Stability

The effect of surfactants on the activity of crude alginase was determined by pre-incubating the enzyme in the presence of Triton X-100 (1%), Tween-20 (1%), Tween-80(1%), SDS (0.1%), Poly ethylene glycol (0.1%), for 30 mins at 40°C before adding the substrate and subsequently relative alginase activities were measured at optimum temperature and alginase activity were determined. To determine surfactants stability, the enzyme was pre-incubated at 40°C for 24 hrs at pH 9. The effect of different surfactants on alginase activity and stability were determined individually for crude alginase of *Bacillus* sp [10].

2.9. Silver Staining

Transfer the gel into fixer containing the following mixture. Water: Acetone: TCA: Formaldehyde keeps in rocker for 5 mins. Wash the gel with water for 3 times (10sec wash) keep in 50% acetone for 5 mins. Add 100 µl of Na₂S₂O₃ (0.1 g/ml). Wash the gel 3 times with millique water. Add 600ml of formaldehyde and 800ml of silver nitrate (0.2g/ml) in 60ml of millique water and keep the gel 3 times with millique water. Add developer to the gel containing following 1.2g of Na₂CO₃, 25ml of Na₂CO₃ (0.1 g/ml), 25ml of formaldehyde in ml of millique water. Develop for 5-10 seconds until bands start appearing. Stop the reaction with 1% Acetic acid [14]

2.10. Immobilization Studies on *Bacillus* sp.

2.10.1. Preparation of Cell Suspension

Bacillus sp. was grown in the production medium. Cells after incubation were harvested by centrifugation at 10,000 rpm for 15 mins. Cells pellet were washed with 2% (w/v) KCl₂ solution, followed by 0.9 % (w/v) NaCl₂ solution and sterile distilled water subsequently. Finally cells were suspended in NaCl₂ solution and stored at 4°C for further studies.

2.10.2. Immobilization of Whole Cells in Sodiumalginate

Cells were harvested after 24 hours by centrifugation at 10000 rpm for 15 mins. Sodium alginate solution for this different concentration of sodium alginate (5, 5.5, 6, 6.5 and 7%) was prepared in boiling water and autoclaved at 121°C for 15 mins. Both alginate slurry and cell suspension (2 ml/10ml of alginate) were mixed and stirred for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into 0.2 M CaCl₂ solution from 5 cm height and kept for curing at 4°C for 1 hrs. The cured beads (mean diameter of 2 mm) were washed 4 times with sterile distilled water after being used as inoculum for alginase production. Approximately 50 beads were transferred to 50 ml production medium with an agitation of 120 rpm at 40°C [15].

2.10.3. Immobilization of Whole Cells in Agar-agar

The various concentrations of agar-agar were dissolved in 18ml of 0.9% sodium chloride solution to get final concentration of 2% and sterilized by autoclaved at 121°C for 15 minutes. Cell suspension (2ml) was added to molten agar-agar maintained at 40°C. Then it was shaken well for few seconds (without forming foam) poured into sterile petriplates and allowed to solidify. The solidified agar block was cut with well puncture (10mm) added to sterile 0.1 M phosphate buffer (pH7) and kept in the refrigerator (1h) for curing. After curing, the agar blocks were washed 4 times with sterile distilled water, after being used as inoculum for alginase production. Approximately 30 blocks were transferred to 50ml production medium with an agitation of 120 rpm at 40°C [15].

2.10.4. Immobilization of Whole Cells in Gelatin

Five milliliters (0.06% DCW) of cell suspension was added to 15 ml of poured into a sterile Petri dish. The gel was over layered with 10 ml of 5% glutaraldehyde for hardening at 30°C. The resulting block was cut into small-size cubes (4 mm) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde, after being used as inoculum for alginase production. Approximately 30 blocks were transferred to 50 ml production medium with an agitation of 120 rpm at 40°C [15].

2.10.5. Statistical Optimization of Nutrient Supplements (CCD AND RSM)

From the optimized nutrient composition for *Bacillus* sp. growth rate, the effect of the pH, temperature, carbon source (Alginate) and nitrogen source (Ammonium chloride) level were studied using Central Composite Design (CCD) [16].

3. Results

3.1. Effect of pH

The effect of pH on alginase stability was determined in pH 9 with various incubation times in mins ranging from 30-180 mins. Maximum alginase activity of 0.77 ± 0.014 IU/ml was recorded in pH 9 at 30 mins. The enzyme activity was decreased to 0.48 ± 0.013 IU/ml in pH 9 at 180 mins was shown in fig. 1.

Figure 1. Effect of pH

3.2. Effect of Temperature

The effect of temperature on the stability of crude alginase was determined in 40°C with various incubation times in mins range from 30-180 mins. Maximum alginase activity of 0.82 ± 0.017 IU/ml was recorded at 30 minutes. The enzyme activity was decreased to 0.24 ± 0.011 IU/ml at 180 minutes. The pH stability of enzyme was measured by the standard assay method with glucose is shown in fig. 2.

Figure 2. Effect of temperature

3.3. Effect of Various Metal Ions

The crude alginase was pre-incubated at 40°C for 30 mins at different concentration of the metal ions. Maximum alginase activity of 0.81 ± 0.016 IU/ml was recorded at calcium chloride. Minimum alginase activity of 0.24 ± 0.009 IU/ml was recorded at potassium chloride is shown in fig. 3.

Figure 3. Effect of various metal ions on alginase activity

3.4. Effect of Different Surfactants

The relative activity of alginase was decreased with increased in concentration of surfactants and also by time of exposure. Maximum alginase activity of 0.51 ± 0.012 IU/ml was recorded at sodium dodecyl sulphate. Minimum alginase activity of 0.2 ± 0.008 IU/ml was recorded at triton X-100 is shown in Fig.4.

Figure 4. Effect of different surfactants on alginase activity

3.5. Silver Staining for Crude Alginase

Figure 5. Silver staining for crude alginase

Crude protein and protein of the pooled fractions from the gel filtration that contained alginase activity after electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel and visualization by silver staining, although a protein band with apparent molecular weight of 125 KDa is shown in Fig. 5.

3.6. Immobilization Studies for Crude Alginase

3.6.1. Production of Alginase by Immobilized Cells of *Bacillus* sp. in Sodium Alginate

Sodium alginate matrix was increased gradually up to 24hrs of incubation after which there were no appreciable changes. The enzyme production reached maximum (0.53±0.012 IU/ml) by 48 hrs is shown in Fig. 6 and plate 1.

Plate 1. Production of alginase by Immobilized cells in sodium alginate

Figure 6. Immobilization of alginase producing *Bacillus* sp. using sodium alginate

3.6.2. Production of Alginase by Immobilized Cells Using Agar Agar

Agar agar matrix was better steadily up to 24 hrs of incubation after which there was no appreciable change. The enzyme production reached maximum level (0.6± 0.017 IU/ml) by 48 hrs with 4%. On further incubation, enzyme production gradually decreased (0.09 ± 0.002) in 7% is shown in Fig. 7 and plate 2.

Plate 2. Production of alginase by Immobilized Cells Using Agar agar

Figure 7. Immobilization of alginase producing *Bacillus* sp. by Agar agar

3.6.3. Production of Alginase by Immobilized Cells Using Gelatin

Gelatin matrix was increased gradually up to 24 hrs of incubation after which there was no appreciable change. The enzyme production reached maximum level in (0.37±0.10 IU/ml) by 48 hrs with 20%. On further incubation, enzyme production gradually decreased (0.19 ± 0.07) in 5% is shown in Fig. 8 and plate 3.

Plate 3. Production of Alginase by Immobilized Cells Using gelatin

Figure 8. Immobilization of alginase producing *Bacillu* ssp. using gelatin

3.7. Statistical Studies for Crude Alginase Producing *Bacillus* sp. Using Central Composite Design

Table 1 shows the five levels of variables chosen for trials in CCD. Response surface methodology (RSM) was used to optimize cultivation conditions for Alginase production, 30 experimental runs with different combinations of four factors and five levels were carried out (Table 2). The variables used for the factorial analysis were pH, temperature, Alginate and ammonium chloride, named X1, X2, X3 and X4 this design, respectively. The effects of the four independent variables on alginase production and the experimental response are along with the predicted response. The interaction effects of variables on alginase production were studied by plotting 3D

surface curves against any two independent variables, while keeping another variable at its central (0) level.

The predicted values from the regression equation closely agreed with that obtained from experimental values. Validation of the experimental model was tested by carrying out the batch experiment under optimal operation conditions. Three repeated experiments were performed and the results are compared. The alginase obtained from experiments was very close to the actual response predicted by the regression model, which proved the validity of the model. At these optimized conditions the maximum alginase production was found to be 36.4 IU/ml. The results show a close concordance between the expected and obtained activity level (Fig.9 and 10a, b, c, d, e and f).

Table. 1. Observed and predicted responses for the experiments performed using CCD design for alginase production

Std	Run	Factor:1 A: pH	Factor:2 B: Temperature	Factor:3 C: Alginate	Factor:4 D: Ammonium chloride	Actual alginase IU/ml	Predicted Alginase IU/ml
12	1	9	50	0.5	0.75	32.5	32.8
9	2	7	30	0.5	0.75	34.3	34.2
24	3	8	40	1	1	31.9	32.1
28	4	8	40	1	0.5	36.4	36.4
7	5	7	50	1.5	0.25	33.5	33.2
5	6	7	30	1.5	0.25	34.8	34.3
30	7	8	40	1	0.5	36.4	36.4
18	8	10	40	1	0.5	33.4	33.4
23	9	8	40	1	0	31.6	31.8
25	10	8	40	1	0.5	36.4	36.4
6	11	9	30	1.5	0.25	33.5	33.1
17	12	6	40	1	0.5	34.7	35.1
3	13	7	50	0.5	0.25	32.8	32.8
15	14	7	50	1.5	0.75	34.4	33.9
27	15	8	40	1	0.5	36.4	36.4
2	16	9	30	0.5	0.25	32.8	33.0
4	17	9	50	0.5	0.25	32.5	32.2
19	18	8	20	1	0.5	32.5	32.8
22	19	8	40	2	0.5	34.4	35.3
16	20	9	50	1.5	0.75	34.3	33.9
8	21	9	50	1.5	0.25	33.2	33.1
29	22	8	40	1	0.5	36.4	36.4
20	23	8	60	1	0.5	31.7	31.9
10	24	9	30	0.5	0.75	32.7	32.6
11	25	7	50	0.5	0.75	33.4	33.3
1	26	7	30	0.5	0.25	34.8	34.7
13	27	7	30	1.5	0.75	34.1	33.9
14	28	9	30	1.5	0.75	33.2	32.9
26	29	8	40	1	0.5	36.4	36.4
21	30	8	40	0	0.5	35	34.6

Table 2. Analysis of variance (ANOVA) for quadratic model for alginate production

Source	Sum of Squares	DF	Mean Square	F Value	P-Value Prob> F
Model	64.56	14	4.61	27.05	< 0.0001 ^c
A-pH	4.17	1	4.17	24.45	0.0002
B-Temperature	1.13	1	1.13	6.61	0.0213 ^c
C-Alginate	0.67	1	0.67	3.91	0.0666
D-Ammonium chloride	0.11	1	0.11	0.63	0.4412 ^c
AB	1.10	1	1.10	6.47	0.0225 ^c
AC	0.30	1	0.30	1.77	0.2027 ^c
AD	0.010	1	0.010	0.059	0.8119
BC	0.64	1	0.64	3.75	0.0717
BD	1.10	1	1.10	6.47	0.0225
CD	0.063	1	0.063	0.37	0.5539
A ²	7.44	1	7.44	43.65	< 0.0001 ^c
B ²	27.89	1	27.89	163.62	< 0.0001 ^c
C ²	3.52	1	3.52	20.66	0.0004 ^c
D ²	32.94	1	32.94	193.25	< 0.0001 ^c
Residual	2.56	15	0.17		
Lack of Fit	2.56	10	0.26		
Pure Error	0.000	5	0.000		
Core Total	67.11	29			

$R^2=0.9594$; $AdjR^2=0.9216$; C.V.%=2.92; ^cmodel terms are significant.

Design-Expert® Software
Alginate

Color points by value of
Alginate:

Figure 9. Predicted response versus actual values selected bacteria for alginate production

Design-Expert® Software

Alginase

X1 = A: pH

X2 = B: Temperature

Actual Factors

C: Alginate = 1.00

D: Ammonium chloride = 0.50

Figure 10a. 3D plot showing the effect of pH and temperature

Design-Expert® Software

Alginase

X1 = A: pH

X2 = C: Alginate

Actual Factors

B: Temperature = 40.00

D: Ammonium chloride = 0.50

Figure 10b. 3D plot showing the effect of pH and alginate

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Alginate

X1 = C: Alginate

X2 = D: Ammonium chloride

Actual Factors

A: pH = 8.00

B: Temperature = 40.00

Figure 10c. 3D plot showing the effect of ammonium chloride and alginate

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Alginate

X1 = B: Temperature

X2 = C: Alginate

Actual Factors

A: pH = 8.00

D: Ammonium chloride = 0.50

Figure 10d. 3D plot showing the effect of alginate and temperature

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Alginase
36.4
31.6

X1 = A: pH
X2 = D: Ammonium chloride

Actual Factors
B: Temperature = 40.00
C: Alginate = 1.00

Figure 10e. 3D plot showing the effect of pH and ammonium chloride

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Alginase
36.4
31.6

X1 = B: Temperature
X2 = D: Ammonium chloride

Actual Factors
A: pH = 8.00
C: Alginate = 1.00

Figure 10f. 3D plot showing the effect of ammonium chloride and temperature

4. Discussion

Microorganism about 30 isolates isolated on the minimal medium supplemented with 0.5% sodium alginate as the sole carbon source were screened. Initially based on the appearance of clearing hydrolytic zone and alginate lyase activities from fermentation culture. It was noted that the pH of the growth medium is an important parameter for bacterial growth and alginase production. The effect of different pH values of growth media was also investigated and it was noted that at pH 8.0 the maximum alginase was produced similarly, the effect of different pH values of growth media was also investigated and it was noted that at pH 8.0 the

maximum alginate (5.71 g/l with 6.83 g/l of DCM) was produced. Increase or decrease in the medium pH resulted in the decreased alginate amount [17].

The existing studies the effects of temperature on alginate lyase activity and stability the maximum enzyme activity of the lyase was observed and was stable at 40°C. Likewise, the lyase possessed approximately 50% activity after incubation at 45°C for 30 mins and was gradually in activated as temperature increased. Further studies on thermal stability at 40, 50 and 60°C with different time periods demonstrated that the enzyme retained more than 60% activity after incubation at 40°C for 3 hrs [18].

Since, we investigated that the effect of addition of metal

ions calcium chloride to increase the activity of alginate lyase. Similarly, Hashimoto *et al.* [19] stated that the effect of metal ions, pH and temperature on the activity of recombinant oligoalginate lyase of *Sphingomonas* sp. MJ-3. In order to optimize the saccharification reaction of alginate polymer, Among the metal ions investigated, K^{2+} , Mg^{2+} , Mn^{2+} , Ca^{2+} and Fe^{2+} displayed activating effects [20].

Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. Immobilization of cells may allow continuous operation of cultivation processes at high dilution rates. Immobilization is a strategy for protecting cells from shear forces many different techniques for immobilizing cells have been proposed [21].

In this study, the maximum alginase production was observed at 48hrs (0.6 ± 0.017 IU/ml). Similar report was observed by Gauthier *et al.* [22]. The experiment on the effect of immobilization using agar-agar showed that the beads gave 4 to 5 folds higher productions over the free cells. The optimized concentration for this matrix was 7%. This report is in accordance with the earlier reported by Anna *et al.* [23], it also matched the use of agar-agar entrapped cells of *Bacillus circulans* ATCC 21783 for cyclo dextrin glucanotransferase production in a fluidized bed reactor led to an enzyme activity (180 U/ml) after 48 hours of incubation. In the present study, the alginase production was studied from 24 to 72 hrs with immobilized cells gelatin and reached maximum (0.37 ± 0.10 IU/ml) at 48 hrs.

In other hand, the natural polymers such as agarose, pectin and gelatin were also employed for cell immobilization of the protease production [24]. In the present study, the protease production was studied by using gelatin as a carrier material. The suitable optimizing concentration for the protease production was 6%. Concurrent to this study, gelatin as a carrier material for the production of β -galactosidase and Penicillin acylase by *E. coli* was carried out by Ramakrishna and Prakasam [25].

RSM was successful applied to the making of keratinase in *Bacillus pumilus* AI, whereas the maximum production was 87.73 U/ml by Zauari *et al.* [26]. Correspondingly, Sivakumar *et al.* [27] stated that the maximum keratinase enzyme production 63.01 U/ml and 60.67 U/ml was obtained by *Bacillus cereus* TS1 and *Bacillus thuringiensis* TS2 respectively. In the present study, it shows that the maximum alginase activity production was 36.4 U/ml. There are four factors namely pH, temperature ammonium chloride and alginate were used for RSM optimization in *Bacillus* sp. Noticeably Sivakumar *et al.* [27] stated that the three factors namely pH, temperature and starch were used for RSM optimization in *Bacillus cereus* TS1 and pH, temperature, mannitol were used for RSM optimization in *Bacillus thuriengiensis* TS2. The factors like glucose soybean and incubation time were used for RSM optimization by Tiwary and Gupta [28]. The variable used by Xian *et al.* [29] were feather meal, soy peptone, sodium chloride, potassium

chloride and potassium dihydrogen phosphate and the factors like sucrose, yeast extract and feather keratin was used by Matsui *et al.* [30]. As a useful statistical technique, RSM has widely and successfully been applied to the optimization of medium components. At present, large research was aimed to isolate feather degrading microorganism and investigated the characterization of feather degrading enzyme for socio-economic importance.

5. Conclusions

Seaweed sample was collected from tide pools and rock surfaces in the kanyakumari coastal region. The selected isolate was to be found more effective for alginase production. At present, many researchers were investigated and characterized the alginase enzyme for socioeconomic importance.

Conflict of Interest

There is no conflict in this study.

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