

Media Optimization for Depolymerization of Alginate by *Pseudomonas aeruginosa* AG LSL-11

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ABSTRACT

An agar degrading bacterium *Pseudomonas aeruginosa* AG LSL-11 was acclimatized to alginate for the production of alginase. Production parameters such as pH, temperature, influence of simple carbohydrates and nitrogen sources, and effect of NaCl on growth and alginase production were carried out. Maximum growth was observed at pH 9.0 and 35 °C, while alginase was produced optimally at pH 9.0 and 30 °C. The alginase produced by *Pseudomonas aeruginosa* AG LSL-11 was inducible by alginate, and repressed by other simple sugar when supplemented along with alginate in the medium. The bacterium did not require NaCl for growth and production of alginase. The activity staining of partially purified culture supernatant after native PAGE revealed the presence of a single alginase.

Keywords: bacterium; *Pseudomonas aeruginosa*; alginase

1. INTRODUCTION

Alginate is a copolymer of α -L-guluronate (G) and its C-5 epimer β -D-mannuronate (M). The monomers of alginate may be arranged as homopolymeric G blocks or M-blocks or alternating GM block structure [Thiang *et al.*, 2000]. Alginate is generally synthesized as cell wall constituent by brown seaweed or exopolysaccharide by pathogen as the virulence factor. Most of the alginate used commercially obtained from three algae belonging to *Macrocystis*, *Laminaria* and *Ascophyllum*.

Alginases also known as alginate lyase or alginate depolymerases, catalyze the degradation of alginate by a beta elimination mechanism of the 4-O-linked glycosidic bond with formation of unsaturated uronic acid containing oligosaccharides [Hee sook and Eun., 2011]. Alginases are used in the production of algal protoplasts and for studying the fine structure of alginate. They have also been used as tools for generating useful oligomeric products of alginate that can be used as therapeutic agents, physiological food sources or plant growth promoters [Thiang *et al.*, 2000]. Hence, increasing attention has been paid to the alginate oligomers formed with alginate lyase, because those oligomers have been found to enhance seed germination, shoot elongation, and root or Bifidobacterium growth [Qing-Da *et al.*, 2008]. Alginate-derived oligosaccharides and their sulphated derivatives are reported to exhibit high tumor inhibition against solid Sarcoma 180 [Hu *et al.*, 2004]. Alginases can be

used in combination with other chemotherapeutics in the treatment of cystic fibrosis (CF) patients infected with alginate-producing *Pseudomonas aeruginosa* [Thiang *et al.*, 2000]. In view of potential applications of alginate lyase in depolymerizing the alginate, increased attention has been paid to produce alginate lyase. The optimization of production conditions for alginate lyase by different bacteria is reported. [Qing-Da *et al.*, 2008] Optimization of culture parameters and growth conditions of *Pseudomonas aeruginosa* AG LSL-11 for the production of extracellular alginate lyase are reported in this article.

2. MATERIALS AND METHODS

2. 1. Materials

Alginate was obtained from Hi-Media Laboratories, India and neocuproine was obtained from Sigma- Aldrich (USA). All other chemicals used in the present study were of analytical grade. Ultra-pure water for all the experiments was collected from Millipore Ultra Purifier (USA).

2. 2. Microorganism and Production of Alginate Lyase

The agar degrading alkalophilic bacterium *Pseudomonas aeruginosa* AG LSL-11, previously isolated in our laboratory by Lakshmikanth *et al.* [2006] was employed for the production of alginate lyase. The bacterium was grown in minimal salts medium containing K_2HPO_4 - 0.38 gL^{-1} , MgSO_4 - 0.2 gL^{-1} , FeCl_3 - 0.05 gL^{-1} , NH_4NO_3 at 1.0 gL^{-1} , and 0.3 % alginate as sole source of carbon. The flasks were incubated on a rotary shaker at 170 rpm at 30°C for 48h. The bacterial cells were removed by centrifugation at 8000rpm for 10 min at 4°C . The supernatant obtained was directly used as a source of crude alginate lyase.

2. 3. Determination of Growth Curve and Alginate Lyase Production.

The cell growth and alginate lyase production in the medium was studied by inoculating the bacterium into the MMS medium with alginate as sole source of carbon. The cell growth and alginate lyase production was measured at an interval of every 12h.

2. 4. Alginate Lyase Assay

Alginate lyase for saccharifying activity was determined by measuring the increase of reducing sugar [Dygert *et al.*, 1967]. One ml reaction mixture containing 0.1 ml of enzyme, 0.5 ml of alginate (0.2 %) and 0.4 ml of 20 mM Tris-HCl buffer (pH 9.0) was incubated for 15 min at 40°C and the reaction was arrested by adding 1ml of alkaline copper and neocuproine reagent followed by heating the contents of the tubes in boiling water bath for 10 mins. The absorbance was read at 450 nm in a UV-visible spectrophotometer (Labomed USA). One unit of enzyme activity was defined as amount that liberates $1\mu\text{mol}$ of glucuronic acid equivalent under assay condition. The alginate lyase activity was also measured by ultraviolet absorption method at 235 nm, in which one unit was defined as an increase of 0.1 in absorbance at 235 nm/min [Qing-Da *et al.*, 2008]. The protein estimation was carried out according to Lowry *et al* [1951].

2. 5. Effect of Temperature and pH on Growth and Alginate Lyase Production

The *Pseudomonas aeruginosa* AG LSL-11 was inoculated into the MMS media supplemented with alginate (0.3 %) and incubated at different temperature from 20 to 40°C ,

cell growth and alginase activity were determined after 48h. Similarly effect of pH on cell growth and alginase production was studied by inoculating 1ml exponential phase culture into alginate containing MMS medium of different pH.

2. 6. Influence of Carbohydrates on Alginase Production

Influence of simple carbon sources on growth and alginase production was studied by supplying simple sugars glucose, fructose, lactose, mannose or galactose at the concentration of 0.1 % (w/v) in the MMS medium with alginate and incubated at 30 °C on rotary shaker at 180 rpm.

The utilization of polysaccharides such as carrageenan, agar, carboxymethylcellulose (CMC) and starch (0.2 %) by the bacterium also tested. Cell growth, and alginase activity in the supernatant were determined after 48h incubation.

2. 7. Nitrogen Sources Favoring Alginase Production.

The 48h grown culture of the *Pseudomonas aeruginosa* in the alginate medium was inoculated in the MMS medium containing 0.3 % (w/v) alginate and one of the different nitrogen sources such as yeast extract (0.1 %), peptone (0.1 %), ammonium nitrate (0.1 %), ammonium sulfate (0.1 %), sodium nitrate (0.1 %) and potassium nitrate (0.1 %). After incubation at 30 °C and 180 rpm for 48 h, cell growth, and alginase activity in the supernatant were analyzed.

2. 8. *In situ* Detection of Alginase

The native-polyacrylamide gel electrophoresis (PAGE) of the partially purified alginase was performed as described by Lammeli., [1970] on 10 % acrylamide slab gel. The *In situ* detection of alginase was carried out according to the method of Gacesa and Wusteman., [1990] by placing native gel on glass plate containing 0.5 % alginate and 1.5 % agar gel and incubated for 30 min at 40 °C, and observed for the clear alginase active band by flooding the alginate-agar plate with 10 % cetyl pyridinium chloride

3. RESULTS

3. 1. Cell Growth and Alginase Production

The time course of growth and alginase production by *Pseudomonas aeruginosa* AG LSL-11 were determined when grown in MMS medium with alginate as carbon source. A typical growth profile and alginase production is presented in Figure 1. The bacterial growth and alginase production were observed after 12h of incubation in the medium, The highest content of reducing sugar resulting from the enzymatic degradation of alginate by *Pseudomonas aeruginosa* AG LSL-11 was observed after 48 h incubation. Production of alginase was also confirmed by plate assay method, a clear zone around the colonies was observed after flooding with 10 % cetyl pyridinium chloride [Figure 2].

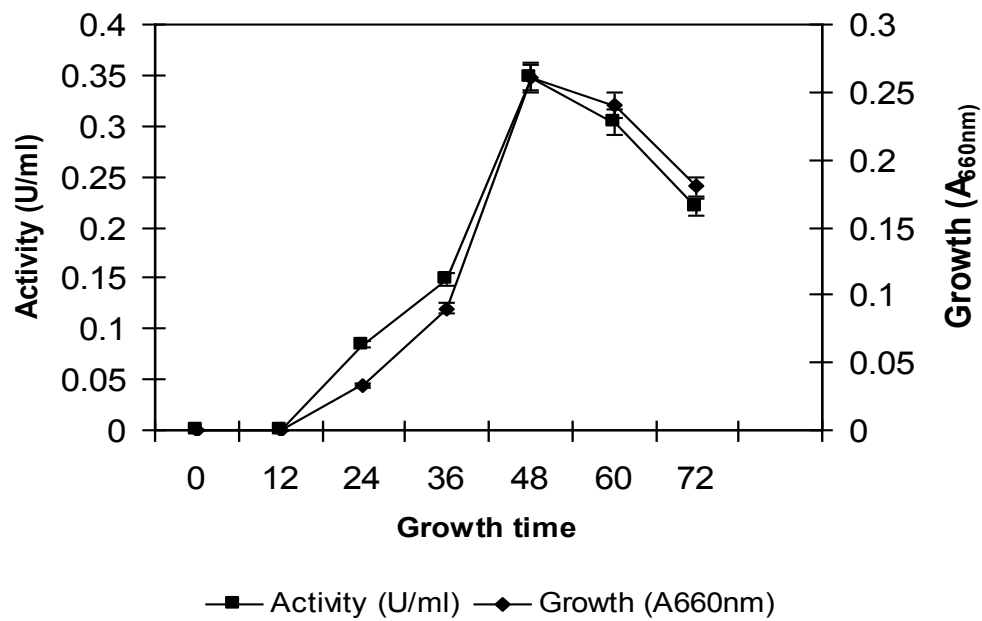


Figure 1. A typical growth curve showing effect of incubation period on growth and production of alginase by *Pseudomonas aeruginosa* AG LSL-11.

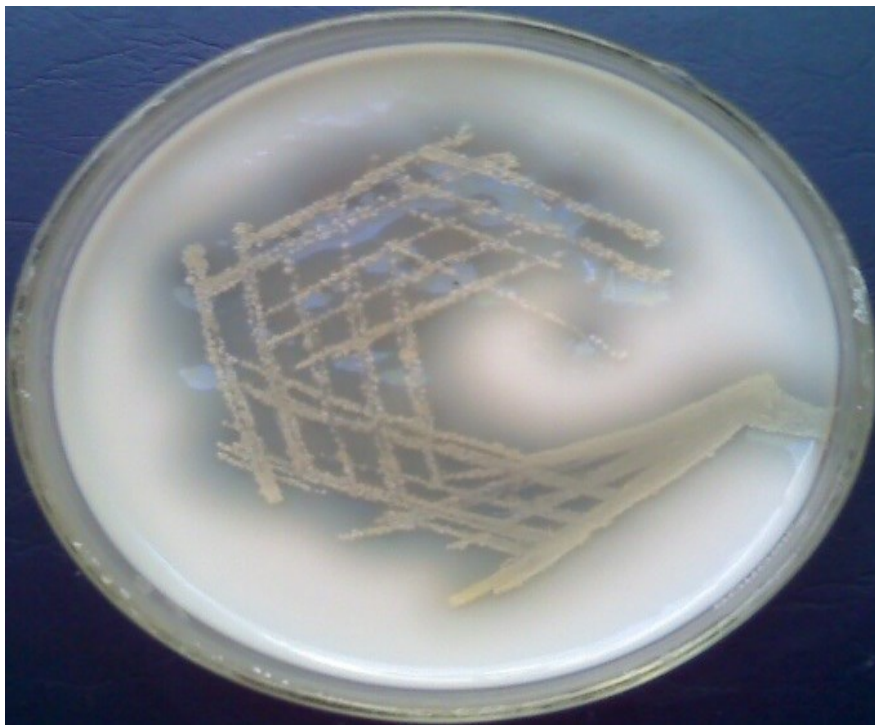


Figure 2. Plate assay for alginase production by *Pseudomonas aeruginosa* AG LSL-11.

3. 2. Influence of Carbohydrates on Alginase Production

Various carbon sources on cell growth and alginase production was evaluated [Table 1]. The maximum alginase activity (0.3481 U/ml) was obtained when the bacterium was grown in the medium with alginate as the carbon source. The simple carbohydrates tested such as mannose, glucose, fructose, lactose and galactose could promote the maximal cell growth. However, only less alginase activity was detected when the bacterium was grown in the medium with those simple carbohydrates as co-supplemental carbon sources. Although, the bacterium was able to utilize other tested polysaccharides, it did not produce alginase.

Table 1. Influence of carbohydrates on alginase production by *Pseudomonas aeruginosa* AG LSL-112.

| Carbon sources | Growth (A_{660nm}) | Activity (U/ml) |
|--------------------------|------------------------|-----------------|
| Alginate | 0.26 | 0.3481 |
| Alginate plus | | |
| Glucose | 0.82 | 0.21 |
| Galactose | 0.70 | 0.15 |
| Fructose | 0.73 | 0.22 |
| Mannose | 0.97 | 0.21 |
| Lactose | 0.55 | 0.22 |
| Carrageenan | 0.35 | ND |
| Agar | 0.53 | ND |
| Carboxy methyl cellulose | 0.19 | ND |
| Starch | 0.44 | ND |
| Xylan | 0.34 | ND |

ND – Not Detected.

3. 3. Influence of Various Nitrogen sources on Alginase Production

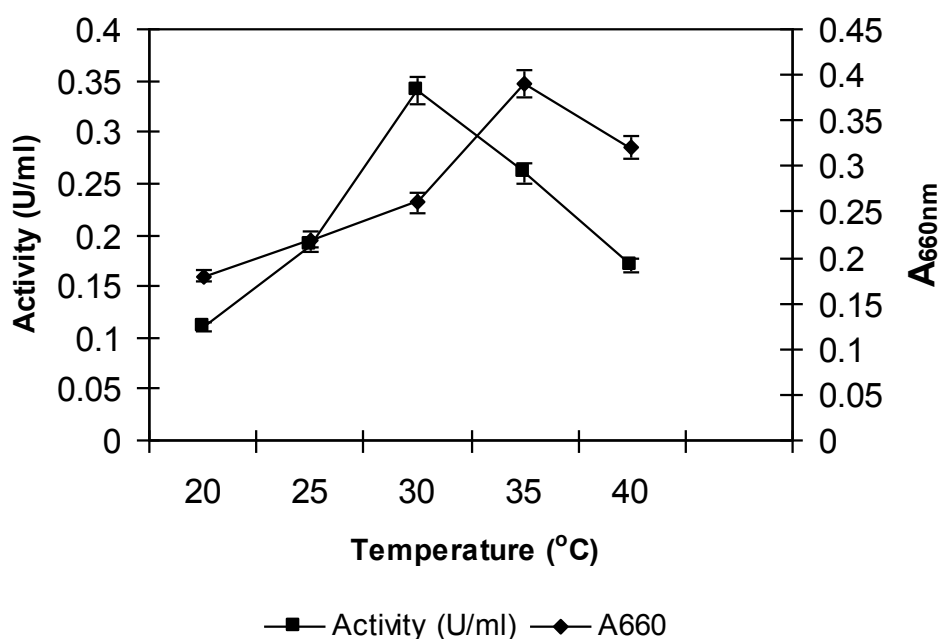
The effect of several nitrogen sources was tested on the cell growth and alginase production and the results are shown in Table 2. All the tested nitrogen sources supported the cell growth when the bacterium was incubated in MMS media with alginate as carbon source. Growth was maximum in the medium which had received organic nitrogen sources. Among the inorganic nitrogen sources, ammonium nitrate promoted the maximum alginase production (0.355 U/ml) after 48 h of incubation. Alginase activity could also be detected in the cultures with all other tested nitrogen sources, but it was lower than that in the ammonium nitrate containing media. Results reveal that ammonium nitrate was the good nitrogen source for alginase production followed by potassium nitrate.

Table 2. Influence of nitrogen sources on alginate production by *Pseudomonas aeruginosa* AG LSL-11.

| Nitrogen Sources | Growth (A_{660nm}) | Activity (U/ml) |
|-------------------|------------------------|-----------------|
| Peptone | 0.4 | 0.037 |
| Yeast extract | 0.38 | 0.029 |
| Ammonium Nitrate | 0.27 | 0.355 |
| Ammonium sulfate | 0.20 | 0.022 |
| Potassium nitrate | 0.26 | 0.244 |
| Sodium nitrate | 0.26 | 0.081 |

3. 4. Effect of Temperature and pH on Cell growth and Alginate Production

Temperature was noted to effect alginate production, the *Pseudomonas aeruginosa* AG LSL-11 grew well in temperature range from 25 to 40 °C. The maximum growth was observed at 35 °C, but at same temperature the alginate production was found to less. The production of alginate was reached maximum (0.355 U/ml) at 30 °C [Figure 3]. The *Pseudomonas aeruginosa* AG LSL-11 grew well in a wide range of pH (6 to 12) and showed good growth at pH range from 7 to 11. Where as the production of alginate was found more in the culture medium at pH between 8 and 10 however, maximum alginate production (0.3481 U/ml) was observed when the medium pH was 9.0 [Figure 4].

**Figure 3.** Effect of temperature on alginate production by *Pseudomonas aeruginosa* AG LSL-11.

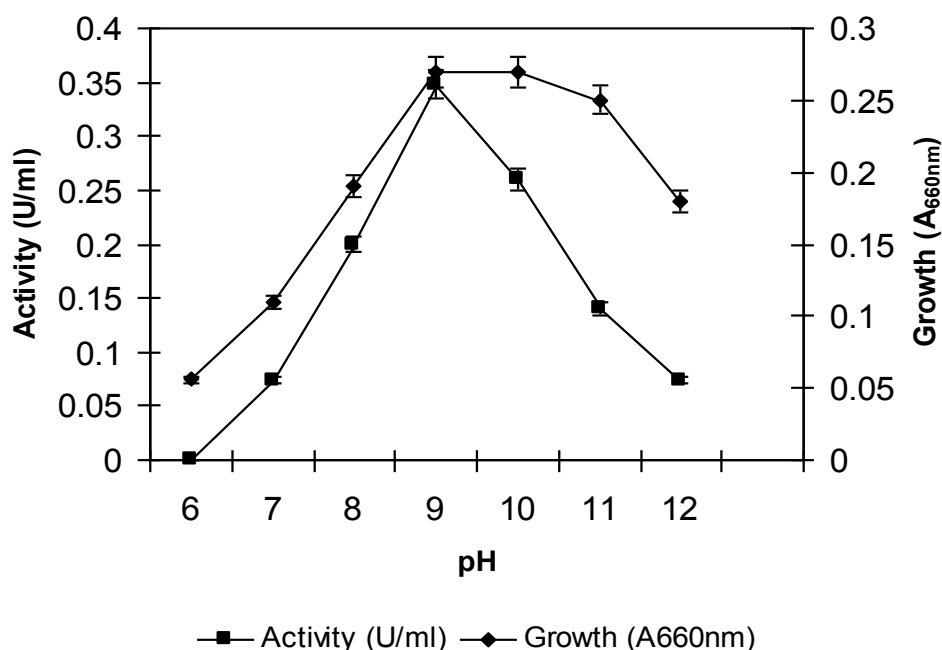


Figure 4. Effect of pH on alginase production by *Pseudomonas aeruginosa* AG LSL-11.

3. 5. *In situ* Detection of Alginase

After the native PAGE the gel was overlaid on 0.5 % alginate containing agar plate and incubated for 30 mins at 40 °C. The native gel was removed and the alginate containing plate was flooded with 10 % cetyl pyridinium chloride, within 5 to 10 mins a clear transparent single alginase activity band was observed [Figure 5].

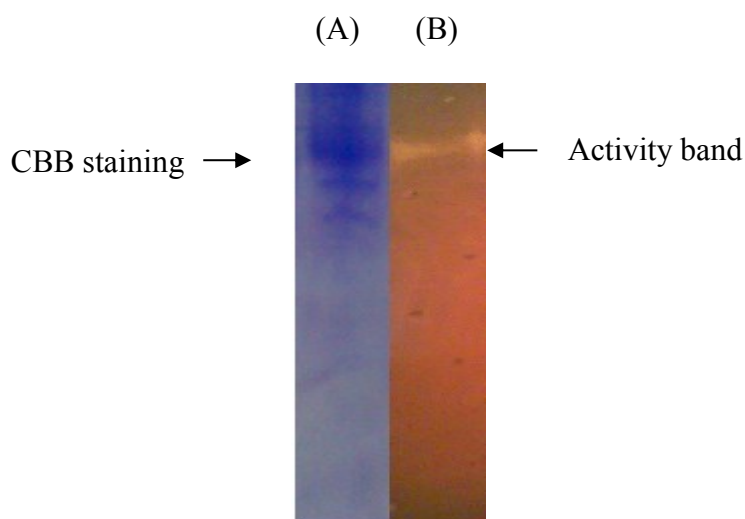


Figure 5. *In situ* detection of alginase (A) Polyacryl amide stained with CBB. B. Agar-alginate gel flood with cetyl pyridinium chloride.

4. DISCUSSION

A unique bacterium *Pseudomonas aeruginosa* AG LSL-11 capable of utilizing various polysaccharides has been isolated in our laboratory. Previously it was utilizing agar as sole carbon source [Lakshmikanth 2006, Basawaraj et al 2012 and 2013], later bacterium was acclimatized to alginate for the production of alginase. A typical growth curve of bacterium reveals that the bacterial growth was observed after 12h of incubation in the medium, also the alginase production in extracellular medium was observed at the same incubation time, both cell growth and the alginase production reached maximum at 48h of incubation. After 48h, the alginase production and cell growth were decreased, this may be due to the unavailability of nutrients (carbon and nitrogen source) to the bacterium in the media. Hence, cell growth as well as alginase production was decreased. Qing-Da *et al* [2008] reported that, the production of alginase by *Flavobacterium Sp* LXA was maximum after 16h. But our results are in agreement with Xiaoting *et al* [2008] who reported alginate lyase production by *Vibrio Sp* YKW-34 at 48h.

The culture conditions for optimal alginase production indicate that *Pseudomonas aeruginosa* grew at the broad pH range of 6 to 11 and optimally at pH 9.0. Most of alginolytic bacteria those were reported till now grew and produce alginase at pH around 7.0, [Preston *et al.*, 2000] and very few near to alkaline pH [Tang *et al.*, 2008]. where as *Pseudomonas aeruginosa* AG LSL-11 grew well and produce maximum alginase at pH 9.0. The results indicates that *Pseudomonas aeruginosa* AG LSL-11 produces alkali tolerant alginase.

P. aeruginosa was able to grow in temperature range from 25 to 40 °C. The maximum growth was observed at 35 °C, but at same temperature the alginase production was found to lower. The production of alginase was reached maximum (0.355 U/ml) at 30 °C. These results are consistent with other reports stating that marine bacteria capable of degrading alginate exhibited optimum alginate lyase activity at around 30 °C (Kim *et al.*, 2010; Li *et al.*, 2011b and Qing-Da *et al*). The *Pseudomonas aeruginosa* AG LSL-11 showed decreased alginase production when incubation temperature was increased beyond 30 to 40 °C.

The simple carbohydrates tested such as mannose, glucose, fructose, lactose and galactose could promote the maximal cell growth. However, only lower alginase activity was detected in the medium with those simple carbohydrates as supplemental carbon sources. The results indicate that the bacterium grown on these simple carbohydrates in conjugation with alginate did not show more enzyme production. This reveals that the enzyme alginase is inducible by alginate in this bacterium and the synthesis of which is repressed by presence of other carbon sources. Similar reports are reported by Alekseev *et al.*, [2004] who observed alginate as good promoter of alginase production by *Pseudoalteromonas citrea* and Qing-Da *et al.*, [2008] also reported the production of alginase induced by alginate alone in the medium. JinYoon *et al.*, [2000] and Doubet and Quatrano, 1982 reported that the alginate lyase specific activity without alginate was reduced by 15-fold, suggesting that alginate is a significant inducer of alginate lyase. All the tested nitrogen sources supported the cell growth in MMS media with alginate as carbon source. Ammonium nitrate promoted the maximum alginase production. Alginase activity could also be detected in the cultures with all other tested nitrogen sources, but was lower than that in the ammonium nitrate containing media. Previous findings suggested that peptone was an important or essential component of growth medium useful for bacterial growth and carbohydrate lyase production [Sugano *et al.*, 1995 and Alexeeva *et al.*, 2002]. Peptone was found to be an unfavorable nitrogen source for

alginate production by *Pseudomonas aeruginosa* AG LSL-11, but peptone supported the maximum growth than other tested nitrogen sources.

The native PAGE gel and *insitu* detection of activity revealed the presence of single alginate enzyme. Few reports are available on multiple alginases in different species. Liyan Li *et al* [2010] reported that three alginases in bacterium *Pseudomonas fluorescens*.

5. CONCLUSION

The *Pseudomonas aeruginosa* AG LSL-11 was successfully acclimatized to alginate for the production of extracellular alginate. The growth conditions were optimized, bacteria produce maximum alginate in presence of alginate and grew well in basic pH i.e pH 9.0 and at 30 °C. The activity staining reveals the presence of single alginate. Hence, the bacterium could be used for the continuous production of alginate.

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