Potential of *Carica papaya* Waste for the Production of Alginic Acid by Fermentation

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Summary: The commercially available alginic acid is mainly extracted from sea weeds. Owing to the growing demand and variation in composition of alginate extracted from different species, there is rising interest in synthesis of alginate by bacteria. Therefore, present research was designed with the aim to check the potential of different papaya wastes like peels, seeds and decayed pulp for alginate production by *Azotobacter vinelandii*. Optimization of physico-chemical parameters was also done. Highest amount of the biopolymer was reported on fermentation of papaya peel at 72 hours of incubation period with inoculum size of 6% (v/v), at pH 7.0, 30^0C and agitation intensity of 200 rpm. Among different carbon and nitrogen sources tested, sucrose and peptone increased the yield of biopolymer (5.34 g/L). Alginate obtained was 98% pure in comparison to the standard. The present research is the first report on utilization of cheap papaya waste for alginate production and will be helpful to save the foreign exchange.

Keywords: Alginate, *Azotobacter vinelandii*, *Carica papaya* waste, Physico-chemical optimization, Fermentation.

Introduction

Alginic acid, the heteropolysaccharides is comprised of β-D- mannuronic acid its C-5 epimer α-L-guluronic acid [1]. The annual production of alginate is approximately 30,000 metric tons. More than half of the produced alginate is utilized in the food industry as a stabilizer, viscosifier, emulsifier, thickener, water binding and gelling agent [2]. Due to its high water absorption capacity, its role as an appetite suppressant has been established and hence can be used to reduce human fat uptake by more than 75%. The biopolymer is also consumed in textile printing, paper industries, manufacturing of ceramics and water-treatment [3]. In pharmaceutical industry, it is consumed in traditional wound dressing, main component of some formulations for preventing gastric reflux and dental impression material. In cell transplantation, alginate immobilized cells are utilized to act as the barrier between the host immune system and the transplanted cell [4].

Commercially, alginic acid is harvested from different brown seaweeds e.g. *Laminaria digitata, Laminaria hyperborea, Ascophyllum nodosum* and *Macrocystis pyrifera* [5]. However, there is difference in structure of alginate isolated from different algal species and sometimes from different tissues of the same plant [6]. Therefore, only limited species of seaweed are found appropriate to be used for alginate extraction. Moreover, this species of sea weed is lacking in Pakistan. Therefore bacterial alginates act as the suitable and promising tool to achieve the desired goals.

At present two bacterial strains are considered appropriate for commercial production of alginate i.e. *Pseudomonas* and *Azotobacter*. Among them, *Azotobacter vinelandii* serves as the best microorganism for biopolymer production,` due to risk of pathogenicity and the less jellifying ability associated with *Pseudomonas* alginate [7]. The overall cost of the fermentation process depends on the substrate used. The expensive pure sugars like glucose and sucrose are commonly used as the carbon source for alginate synthesis by fermentation [8]. Pakistan is an agricultural country and produces about 50-60 million tons of agricultural waste annually that becomes the major cause of environmental pollution. Hence, there is growing trend towards the utilization of these nutritionally rich by-products for the production of alginate. According to the literature, presently there is only one report on the use of wheat bran for the biopolymer synthesis [3]. Due the increasing demand of alginate in the country, the research was planned to explore the potential of *Carica papaya* waste for the production of alginic acid using *Azotobacter vinelandii*. It will be helpful to fulfill the required demand, save the foreign exchange spend on import of alginate from developing countries and reduce the environmental pollution by utilizing the waste product for useful product formation.

Experimental

Microorganism and culture maintenance

The strain of *Azotobacter vinelandii*, NRRL-14641 was present in the Institute of Biochemistry

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and Biotechnology, University of Veterinary and animal Sciences, Lahore. The bacteria was cultured and preserved on Burk's Nitrogen free agar medium slants [3].

Inoculum preparation

A.vinelandii was transferred from Burk's Nitrogen free agar medium plates to Burk's Nitrogen free medium (25 mL) contained in Erlenmeyer flask (250 ml). Further, the culture was incubated in orbital shaker for 24 hours at 30°C and 200 rpm till optical density of 0.6 was achieved at 600 nm. This broth was utilized as an inoculum for further experimentation [1].

Substrate collection and proximate analysis

Papaya waste including peels, decayed pulp and seeds was collected from the local market of Lahore, Pakistan. The substrate was milled and sieved to obtain the size of 0.1mm. The moisture content, crude protein, crude fat, crude fiber and ash were estimated by AOAC method [9].

Medium preparation and optimization of parameters for hyper-production of alginate

For fermentation studies, the basal media contained (g/L): 1.5% $CaCl_2$, 2% $MgSO_4$ and 2% corn steep liquor. Different papaya wastes (peel, seed and decayed pulp) at 7.5% (w/v) were used as carbon source to select the best substrate for hyper-production of alginic acid. The flasks were autoclaved and inoculated with 2% (v/v) inoculum pH 7.0, 35^0C and agitation speed of 200 rpm at different incubation time periods (24- 120 hours) to select the optimum time period for product formation [3]. Then percentage of inoculum (2- 10%), pH (5-10), various degrees of temperature (25- 50ºC) and agitation speed (120- 280 rpm) was optimized to obtain maximum exopolysaccharide secretion at optimized incubation time. Effect of addition of different carbon sources (glucose, fructose, sucrose, maltose and lactose) and organic nitrogen sources (casein, yeast extract, corn steep liquor and peptone) on biopolymer production was studied [1].

All the conditions were optimized in Erlenmeyer flasks (250 mL) containing 25 mL total fermentation medium using one factor at a time approach.

Extraction and estimation of Alginate

For extraction of exopolysaccharide, 1 mL of (0.5M) EDTA sodium salt solution and 0.5 mL of (5.0M) NaCl solution was added to the fermentation media. Then to separate the biomass, the solution was centrifuged at 18000 rpm, 20^0C for 30 minutes. Further, the supernatant was cooled in an ice bath and ice cold isopropanol (three times) added. The solution was placed at 4^0C for 24 hours. The mixture was again centrifuged for half an hour at 18000 rpm, 4^0C for precipitation of alginate. The precipitate obtained was suspended in water, centrifuged and finally the precipitated alginate was dried in oven for 24 hours at 80ºC. The dried precipitate of alginic acid was finally weighed and reported (Butt *et al.* 2011). Alginate was identified by FTIR (Shimadzu/ Prestige-21) and quantified by HPLC method [10] using the standard of Sigma-aldrich. The guluronic acid to manuronic acid ratio was estimated through colorimetric reaction with carbazole reagent at 546 nm [11].

Statistical analysis

The shake flask studies were carried out in triplicates. The standard error values have been displayed as Y error bars in graphs. The data was analyzed on SPSS 13.0 software, by comparing mean through One-Way ANOVA and multiple comparison was made through LSD and Descriptive analysis [12].

Results and Discussion

Proximate analysis of the substrate

The results of the proximate analysis of the different wastes of *Carica papaya* waste are presented in Table-1.

Table-1: Proximate analysis of the substrate.

Composition (%)	Seed	Pulp	Peel
Moisture	40.51	88.25	67.39
Crude Protein	2.57	1.17	6.89
Crude Fibre	2.09	0.93	9.36
Crude Fat	3.27	0.37	0.33
Ash	4.27	0.45	3.15
N.F.E	47.29	8.83	12.88

Screening of best fermentation media and incubation time for production of alginate

Among the different parts of papaya waste tested, the highest ($p<0.05$) amount of alginate (2.45 g/L) was achieved on fermentation of peels after 72 hours of incubation time followed by papaya seed (1,98 g/L) and decayed pulp (1.6 g/L) under similar set of conditions (Fig. 1). Papaya peels serve as the nutritionally enriched media for the production of the biopolymer, as it contains highest amount of crude protein and different essential nutrients. This is the

first report on the production of alginate from papaya waste. Maximum alginate concentration (7.46 g/L) by *A. vinelandii*, was observed at 7.5 % wheat bran after 48 hours of incubation time under optimized conditions Saeed *et al.* [3]. Butt *et al.* [1] reported incubation period of 110 hours as optimum for the exopolysaccharide synthesis by both the wild and mutant strain of *A.vinelandii*. Khanafari and Sepahei [13] contrasted with the present outcomes and stated that the greater yield of alginate (5 mg/mL) was observed at 24 hours of incubation time by *Azotobacter chroococum*1723, using whey as substrate whereas Emtiazi *et al.* [14] reported the highest production (7.5 mg/mL) by using sucrose (1%) and beet molasses (2%) in fementation medium by *Azotobacter* AC2 after incubation at four days. Ali *et al*. [15] used wheat bran as the substrate for solid state fermentation of alginate and reported maximum yield (8.8 g/L) at six days of incubation period. Thus it is concluded that *carica papaya* peels have potential to be used as cheap substrate for alginate production in comparison to the costly pure sugars already used.

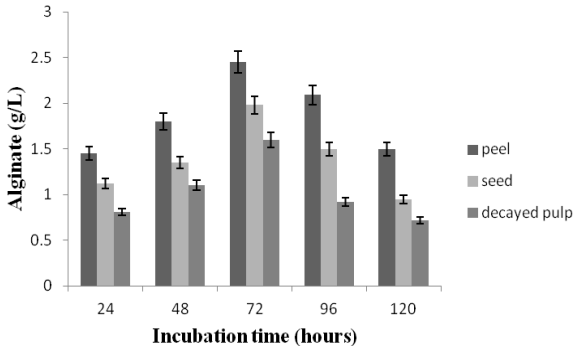

Fig. 1: Screening of the best substrate for production of alginate by *Azotobacter vinelandii.*

Effect of inoculum size

Different inoculum volume (2-10%) were tested under pre-optimized conditions and the significantly ($p<0.05$) highest amount of exopolysaccharide (3.12 g/L) was produced at 6% volume of inoculum (Fig. 2a). The significant results of optimization of incubation time are in-line with similar experiment carried out using agricultural waste, as maximum concentration of alginate (7.46 g/L) was reported at 6% inoculum size [3]. These results are against the results of Vermani *et al*. [16] as 2% inoculum volume was suggested to give the best yield (8.25g/L) by *A.vinelandii* MTCC 2460.

Effect of pH

The effect of varying levels of pH was examined and the results indicated maximum secretion at pH 7 (3.12 g/L) as shown in Fig. 2b. The results are in-line with results of different scientists as they reported pH 7 to be optimum pH for alginate production [1, 3]. The results of Pandurangan *et al*. [17] differed with the present data as they found pH 8.0 to be optimum for exopolysaccharide synthesis using *Azotobacter chroococcum* (yield %= 46.64)..

Effect of temperature

Various degrees of temperature (25 to 50^0C) were optimized and 30^0C was found to give significantly (3.61 g/L) best production (Fig. 2c). The results obtained were in agreement with previous scientists [1,3]. In contrast, Pandurangan *et al.* [17] reported 34 ^{0}C to be the optimum temperature to obtain best exo-polysaccharide yield by *Azotobacter chroococcum* (45.63%).

Effect of agitation speed

Various agitation intensities (120 to 240 rpm) were tested and the significant ($p<0.05$) maximum yield (3.61 g/L) was obtained at 200 rpm (Fig. 2d). The results of optimization of agitation speed were in agreement with literature [1, 3, 14] as they reported that the enhanced production of alginate was achieved at agitation intensity of 200 rpm.

Effect of carbon source

Effect of addition of different carbon sources were tested to obtain maximum amount of alginate. The significantly highest ($p<0.05$) amount of alginate (4.05 g/L) was observed by addition of sucrose in the fermentation media (Fig 3a). The results are in line with Butt *et al*. [1] and thus suggest that sucrose is the most readily metabolized sugar by the bacteria for the production of an exopolysaccharide.

Effect of organic nitrogen source

From the various organic nitrogen sources tested, peptone gave the highest titer (5.34 g/L) of alginate (Fig 3b). Both the positive and negative effect of source of nitrogen addition on alginate production is reported in literature. However, the results of our present study correlate with the study of Saeed *et al*. [3] as they reported that addition of corn steep liquor enhanced the yield of alginate. The

similar pattern was observed by Butt *et al*. [1] as they reported positive effect of peptone on biopolymer production. The results of the present research are also supported by Galal and Ouda [18] as they analyzed the maximum alginate concentration at 0.6% yeast extract and 0.8% corn steep liquor by *A. chroococum* isolates n.1 and n.8 respectively. Pandurangan *et al*. [17] also reported positive effect of addition of yeast extract on alginate production.

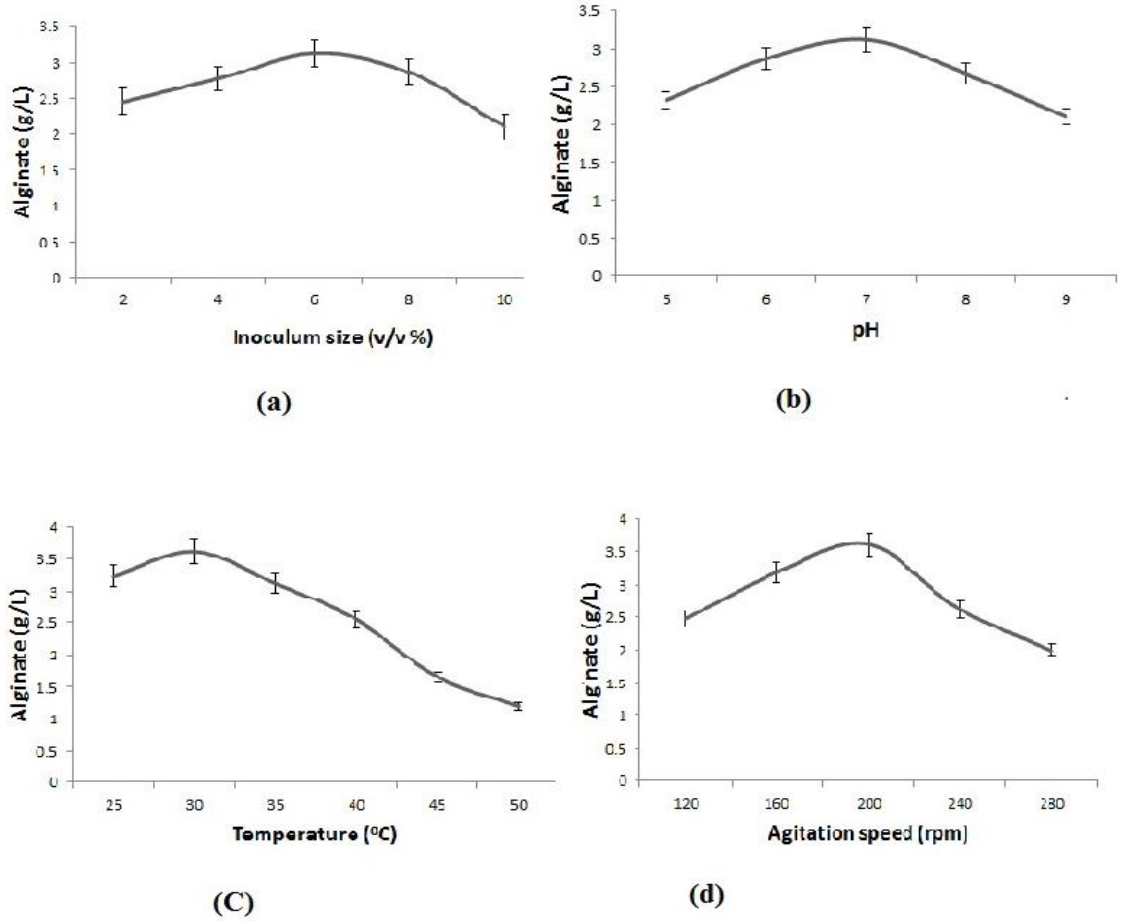

Fig. 2: Optimization of physico-chemical parameters for hyper-production of alginate by *Azotobacter vinelandii*. (a) Incubation time (b) pH (c) Temperature (d) Agitation speed.

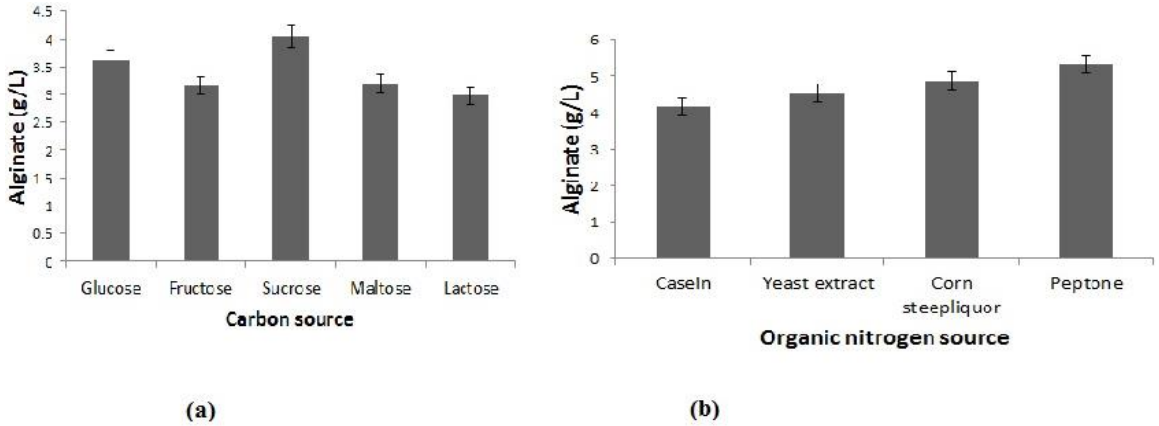

Fig. 3: Effect of (a) carbon and (b) nitrogen source on alginate production.

Identification and quantification of alginic acid

The identification of alginate was done by FTIR against the standard (Fig 4). The product was 98% pure in comparison to the standard as detected by HPLC method (Fig. 5).The guluronic acid to mannuronic acid was found to be 0.43 (33% guluronic acid and 77% mannuronic acid) while of algal alginate was 0.81 (45% guluronic acid and 55% mannuronic acid) as shown in Fig. 6 .

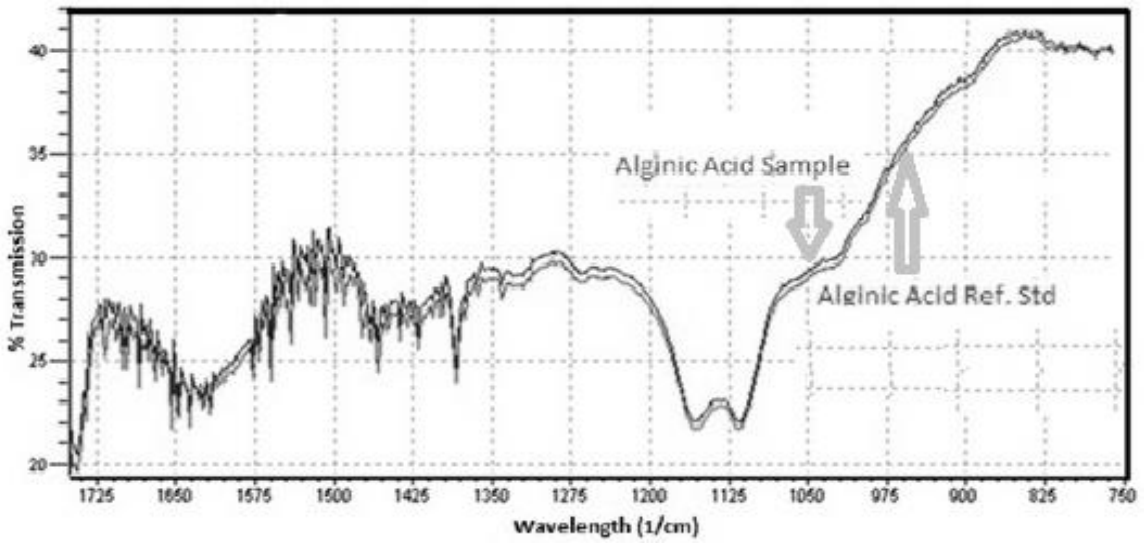

Fig. 4: FTIR spectrum of alginic acid.

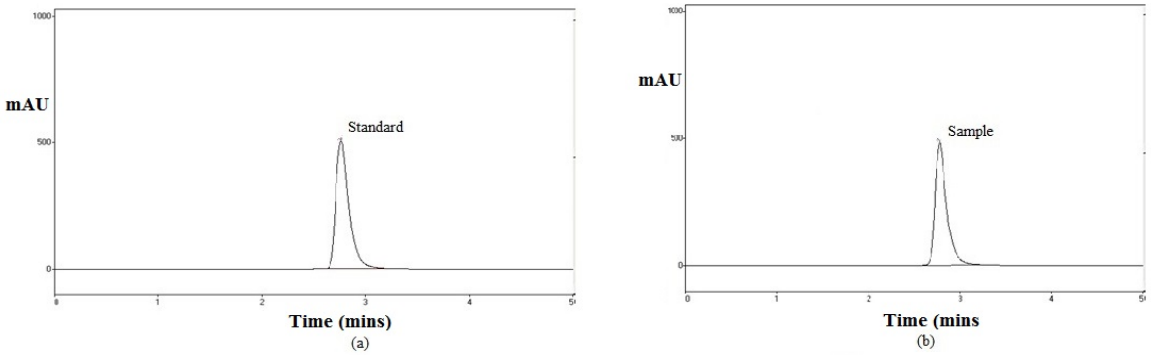

Fig. 5: HPLC chromatogram of alginic acid (a) Standard (b) Sample.

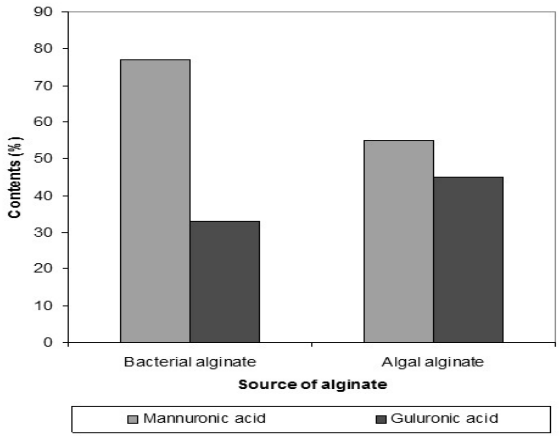

Fig. 6: Comparison of mannuronic acid and guluronic acid contents of the bacterial and algal alginate.

Conclusion

The outcomes of the present study revealed that the *Carica papaya* waste has the potential to be used as substrate for the production of biopolymer i.e. alginate. The optimized conditions can be further used for the commercial production of alginic acid on the commercial scale.

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