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Optimization of alginate production from diazotrophs by mutagenic approach

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Abstract
Nowadays, all commercial alginates are extracted from brown seaweeds, and only two bacterial genera have been recorded to secrete alginate, *Azotobacter,* and *Pseudomonas*. In the present study, the bacterial strains *Bacillus licheniformis PS01, Paenibacillus Riograndensis PS02, Pseudomonas fluorescence PS03* isolated from North Gujarat region from the rhizosphere of *Triticum, Momordica charantia, and Nicotiana tabacum*, respectively were used. All the bacterial strains were recognized to produce alginate biopolymer, hence to increase the alginate yield mutation study was carried out. The mutational study was done using physical parameter ultraviolet (UV) radiation (exposure at a 254nm wavelength for a different time interval) and chemical mutagens, Benzalkonium chloride (BKC) (50µl/ml to 500µl/ml) and Ninhydrin (0.1µg/ml to 10µg/ml). Characterization of the extracted alginate biopolymer from the wild-type strains was done by FE-SEM, FTIR, HPLC, and GPC. Results confirmed that the PS01-Nin1 gives the highest production of alginate 11.04 $g.L^{-1}$ at 0.1 µg/ml ninhydrin concentration, PS02-Nin2 (0.5 µg/ml) and PS02 both gave similar results (8.82 $g.L^{-1}$), and PS03-UV3 gave 10.66 $g.L^{-1}$ highest alginate concentration after the exposure of 3 min to UV radiation. Characterization confirmed the presence of alginate biopolymer with 2.3 x 10^3kDa, 2.2 x 10^3kDa and 2.2 x 10^3kDa higher molecular weight from PS01, PS02, and PS03 strain respectively.

Keywords: Alginate, Bacillus *licheniformis, Paenibacillus riograndensis, Pseudomonas fluorescence*

Introduction
Increasing research on the biopolymer production by bacteria led to improving the production process and applications in new and diverse fields [1]. One of the most significant advantages of using biopolymers is its biodegradability, which makes them a renewable product. However, higher cost of bacterial alginate is a significant drawback [2].
Microorganisms can produce different kinds of polymers as a reserve storage polyesters by utilization of simple to complex substrates under favorable growth conditions [3, 4]. Usually, these biopolymers accomplish similar function whereas others are specific for other species and perform distinct biological functions. By cellular location, biopolymers could be either intracellular or extracellular. The intracellular polymers are few and can be of limited use. While extracellular polysaccharides are vast in range and have been grouped into four class, i.e. polysaccharides, polyamides, polyesters and inorganic polysaccharides [5]. The primary purposes of the exopolysaccharides (EPS) are adherence to the surfaces, protection from the engulfment by predators, protection from the environmental changes and migration of prokaryotes in groundwater [6]. Alginate, xanthan, dextran, and cellulose are the excellent examples of exopolysaccharide [7]. We are focusing on alginate in this study.
Alginate, biopolymer typically extracted from the marine brown algae, Phaeophyta [8, 9]. This is present in the cell as a structural component of the cell wall and intracellular spaces in algae and capsular material in some bacteria [9]. *Macrocystis pyrifera, Laminaria, Ecklonia and Ascophyllum Nodosum* are the different algal species that are known to produce commercial alginate biopolymer [10, 11]. Alginate is also produced as an EPS by opportunistic pathogen *Pseudomonas* and nitrogen-fixing aerobic diazotroph *Azotobacter* species [12, 13, 14, 15]. However, alginate obtained from *Pseudomonas* has the poor jellifying capacity [16]. Hence, research on alginate production by non pathogenic bacteria is on demand.
Alginate is a negatively charged non- repeating, an unbranched linear polysaccharide composed of (1– 4)-β-D-mannuronic acid (M- residues) and its C-5-epimer α-L guluronic acid (G-residues) [17, 11]. Alginate has the common molecular formula $(C_6H_8O_6)_n$ with the molecular mass varies between 10,000 to 6,00,000 [10]. The high molecular weight of bacterial alginate and the negative charge on it ensures that the polysaccharide is highly hydrated and viscous [18].

Clementi (1999) reported molecular weight 168 ± 58 kDa after the incubation of 140 h [19]. Pena *et al*., (1997) recorded 1400 to 2000 kDa after sucrose fermentation for 72h [20]. Anyanee P documented 0.77 x 10^3 to 6.75 x 10^3kDa molecular weight of alginate extracted from *Azotobacter* spp [18].

The importance of alginate is based on their vast industrial applications as thickening agents, emulsifiers in food, gelling agents, stabilizers, paper making industries and textiles [21, 22]. Alginates have broad applications in pharmaceutical and medical devices due to its properties like biocompatibility, biodegradability, and safety. It has been used in inflammatory agents, wound healing, radioactive suppressive agents, carriers for delivering chemical drugs and proteins [23, 24]. Furthermore, it is also vital in detoxifying wastewater and metal pollution [25, 14]. Alginates have some applications in cell cultures (alginate gels are being used as scaffolds for 2D and 3D culture systems) and in tissue generation (used as cell adhesion carriers of DNA, antibodies, and proteins; regenerative properties) [26, 27].

However, the properties of alginate depend on climatic conditions, environmental changes and the type of algae. Whereas the chemical properties of alginate rely on four different factors; 1) chemical composition of it monomer M and G residues and its position of epimer, 2) molecular weight of the resulting polysaccharide chain, 3) sequence pattern of monomers; and 4) acetylation degree [28, 29]. Change in alginate properties can be achieved by manipulating the bacterial strain by creating mutation through various mutagens and changing the experimental parameters. Although a large number of bacterial biopolymers are investigated very few, have been commercialised. Hence, screening of different biopolymer producing bacteria using different substrates is necessary.

Very few studies have been reported for increasing the alginate concentration by changing the strain by mutation. Butt, Haq,and Qadeer (2011) reported that mutant *Azotobacter vinelandii* EMS-45 strain gave 1.55 fold better production of alginate than *Azotobacter vinelandii* NRRL-14641 parent strain [30]. They used three different mutagens (UV irradiation, Nitrous acid (HNO2, 0.1- 0.4M); and, ethyl methane sulphonate (EMS, 25- 100µl/ml). Study on *Azotobacter vinelandii* AT268 mutant in poly β hydroxybutyrate production due to mutation in phbR gene (transcriptional activator of phbBAC biosynthetic operon); and mutant CNT 26, mutation in much 26, increase the transcription of gene algD (codes for GDP mannose dehydrogenase, enzyme responsible for alginate biosynthesis) confirmed 25% less alginate production than the parent wild-type strain [31] (Pena *et al*., 2002).While the strain having a mutation in both the genes phbR and muc26 reported producing alginate with the highest molecular weight 4.0 x 10^6 Da with the very low polydispersity index (1.3) [31]. However, Meija *et al*. (2009) concluded that the *Azotobacter vinelandii* AT6 strain (mutant in Poly β hydroxybutyrate production) gives two-fold higher alginate concentration 9.5 g l^{-1} than wide type strain (ATCC 9046) using two-stage fermentation process [32]. A recent study demonstrated that the parent strain *Azotobacter vinelandii* NRRL- 14641 when mutated randomly by ethidium bromide treatment increase the production of alginate by 2.11 times than the wild-type strain [33]. They have used three mutagens; ultraviolet irradiation, Nitrous acid, and ethidium bromide.

Here, due to the growing demand of alginate, the present study was carried out to maximize alginate yield by mutant strains of *Bacillus licheniformis* PS01 strain, *Paenibacillus riograndensis* PS02 strain and *Pseudomonas fluorescence* PS03 strain. The strains were mutants using three different chemical and physical mutagens: physical mutagen; Ultraviolet rays; and chemical mutagen; ninhydrin and Benzal Konium Chloride.

Materials and Methods

Microbial strains, maintenance, and storage

Bacillus licheniformis PS01 strain (Parent Strain) (NCBI Genbank accession number MG430800), isolated from soils of *Triticum* from latitude 23°33'12.87"N and longitude 72°43'56.77"E of Vijapur village of Mehsana District, Gujarat, India. *Paenibacillus riograndensis* PS02 strain (Parent strain) (NCBI Genbank accession number MG432472), isolated from the rhizosphere of *Momordica charantia* from Village Tareti, Mehsana, Gujarat, India (Latitude-23°37'59.41"N, Longitude-72°23'38.89"E). *Pseudomonas fluorescence* PS03 strain (Parent Strain) (NCBI Genbank accession number MG397070), isolated from the rhizosphere of *Nicotiana tabacum* plant from village Talod, Sabarkantha, Gujarat, India. The strains were preserved in the sterile soil as well as on Jensen's medium slants (containing 20g Sucrose, 1.0g Dipotassium phosphate, 0.5g Magnesium sulfate, 0.5g Sodium chloride, 0.1g Ferrous sulfate, 0.005g Sodium molybdate, 2.0g Calcium carbonate, 15g Agar) [34].

Inoculum Preparation

One loopful of bacterial culture was transferred to 25 ml of alginate producing medium (AP) (containing 20.0 g sucrose; 0.6 g $(NH_4)_2SO_4$; 2 g Na_2HPO_4; 0.3 g $MgSO_4.7H_2O$; 6 g Yeast Extract) in 100 ml flask [35]. The flask was then incubated at 30°C for 24 hours. The bacterial culture is having a final density of 3 X 10^8 cells per ml was used as inoculum [33]. 10 ml of the final density (3 X 10^8 cells per ml) inoculums was centrifuged to pellet out the cell biomass. The cells were washed thrice with 0.2 M phosphate buffer (pH 7.0) to remove traces of medium components adhered to the cell surface. The final obtained cells were used for the further process.

Mutagenesis

For strain improvement, the above parent's strains were subjected to various chemical (Ninhydrin and Benzalkonium chloride) and physical mutagens (i.e., UV irradiation).

Physical mutagen- UV Irradiation

For UV mutagenesis, the phosphate buffer washed cells were resuspended in 1 ml of phosphate buffer and then exposed to UV rays for a different time interval (30 seconds – 20 minutes). The distance between UV lamp and the bacterial culture was 5cm to obtain more than 90% kill rate. UV exposed cells were kept inthe dark to prevent photoreactivation and further transferred to AP broth. Parent strains (auxotrophs) were also screened for their alginate production ability [30].

Chemical mutagen- Ninhydrin

Finally, 0.1 to 1.0 µg/ml of different ninhydrin (CAS Number 485-47-2, Sigma Aldrich) concentration was prepared from the phosphate buffer washed cells. The cell suspension with ninhydrin mutagen was incubated at 30°C for 30 min. Cells were centrifuged and washed with phosphate buffer to remove

the traces of mutagen. Finally, cells were resuspended in 1ml of phosphate buffer. 0.2 ml of suspension was inoculated in 25 ml AP broth and incubated at 30°C for PS02 and PS03 strains, 37°C for PS01 strain for 96 hours at 120 rpm.

Chemical mutagen- Benzalkonium chloride (BKC)
The cells were subjected to mutagenesis by adding different concentrations of BKC (CAS no 63449-41-2; Sigma Aldrich), i.e. 50 to 500 µg/ ml. The culture was incubated at 30°C for 30 min. The same procedure was followed as mentioned above (refer to ninhydrin mutagenesis).

Analytical determinations
For the quantitative determination of alginate concentration, 10 ml of culture broth was withdrawn after 96 hours of incubation. After the addition of 1ml, 0.5 M EDTA sodium salt solution (to solubilize cell associated alginate) and 0.5 ml of 0.5M NaCl solution the mixture was subjected to centrifuged at 8000 rpm at 15°C to separate cell biomass in a pre-weighed centrifuge tube [17]. The supernatant containing solubilized capsular material and exopolysaccharide was separately treated with three-foldice cold isopropanol in a different preweighed tube. The mixture was left at 4°C for overnight and then subjected further for centrifugation precipitates were dried at 80°C for 24 hours and weighed. The cell biomass pellet was washed at 8000 rpm for 45 min. Pellet was washed with distilled water and with distilled water and weighed.

Characterization of Alginate
Field Emission- Scanning electron microscopy (FD-SEM) and elemental analysis
FD-SEM did surface morphology study of extracted alginate compound. JEF 7100 F (oxford incorporation). Elemental analysis was done using dispersive energy X (EDX) ray spectroscopy. The 0.01 wt% solution of the sample was prepared in 10 µl miliQ water. The solution was carefully poured onto copper stub and allowed to dry in an oven at 60°C and gold coated with sputter coater.

Fourier-transform infrared spectroscopy (FT-IR)
FT-IR spectra were obtained using Nicolet 6700 FT-IR spectrometer. All the spectra were recorded at room temperature. For recording spectra, a cell with KRS-5 windows and Teflon spacer was used; the optical path length was 1 cm. For each spectrum, 40 scans were made with a selected resolution of 2 cm^{-1}.

High-Pressure Liquid Chromatography (HPLC)
Shimadzu Prominence HPLC system was used for the analysis equipped with an RI detector. C18H 5µm 150 mm x 4.6 mm column used. 5mM $H_2SO_4$was used as a mobile phase [36]. The mobile phase flow rate was 0.5 ml,and the temperatures of the column and detector were set at 40 respectively. Samples and solutions were prepared in 0.05% in 10mM phosphate buffer (pH-7.2).

Molecular weight determination
Alginate molecular weight was determined by high temperature - gel permeation chromatography (HT-GPC) using a Waters 2695 Separation Module equipped with a 2414 RI detector and having Ultrahydrajel 500 and 120 columns in series. Columns were eluted with 0.1 M aqueous $NaNO_3$ at a flow rate of 0.5 ml min^{-1}. Calibration was performed using a dextran standard ranging from 401 000 to 4400 peak molecular weight. The concentration of solutions was 0.01 wt% in water [20].

Result and Discussion
The vegetative cells of PS01, PS02 and PS03 strains (wild-type) were exposed to physical mutagen (UV irradiation) (Fig. 1) for different time intervals and chemical mutagen (Ninhydrin and BKC) (Fig. 2 and Fig. 3, respectively) at different concentrations to isolate a mutant with enhanced alginate production ability with more than 90% kill rate. The survivors were tested for its ability to produce alginate (exopolysaccharide) biopolymer.

UV mutagenesis
To check the effect of UV radiation on PS01, PS02 and PS03 strains, bacterial isolates were exposed for a different time such as 30sec, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20 minutes. PS01-UV1 (after mutation strain) shows highest alginate production (7.76 $g.L^{-1}$) after the exposure to UV irradiation for 1 minute (Fig. 1). Though PS01gives higher production then PS01-UV1 (8.8 $g.L^{-1}$). PS02-UV2 (after mutation strain) shows a negligible and constant increase in alginate production depending on the UV exposure time. PS02-UV2 strain was observed to increase the production for 2.17 fold (PS02-UV2; 5.66 $g.L^{-1}$, PS02; 2.6 $g.L^{-1}$) than the parent strain PS02 (Fig. 1). Whereas, PS03-UV3 strain gave the incredible results by increasing the alginate production 2.6 fold (PS03-UV3; 10.66 g.L-1, PS03; 4.1 g.L-1) after the exposure of 3 minutes to UV radiation than PS03 (Fig. 1). This might be due to the production of thymine-thymine dimmers which resulted from the mutation [37, 38].

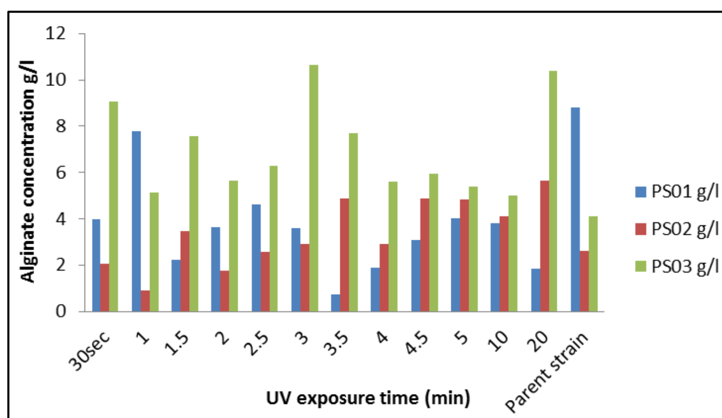


Fig 1: Effect of UV irradiation on PS01, PS02 and PS03 strains alginate production ability after 96 hours of incubation on a rotary shaker (120 rpm)

Ninhydrin mutagenesis

Different concentrations of Ninhydrin (0.1 to 1 µg/ml) were taken to evaluate its effect on alginate production ability of PS01, PS02 and PS03 strains (Fig.2). In comparison with the UV mutagenesis and parent strain PS01, PS01-Nin1 (after ninhydrin mutation strain) gives higher alginate production (11.04 $g.L^{-1}$) (1.29 fold, 1.46 fold, respectively) with 0.1µg/ml ninhydrin concentration. Similarly, PS02-Nin2 also gives 1.27 fold higher alginate production (8.82 $g.L^{-1}$) than PS02. While the *Pseudomonas* species PS03 straindoes not show any remarkable increase in alginate production after the mutation (PS03- Nin3 (0.1 µg/ml ninhydrin); 6.38 $g.L^{-1}$, PS03; 5.16 $g.L^{-1}$). This might be due to the reason that when ninhydrin reacts with the aminoacids present in the cell protein results in the separation of water molecule and formation of Schiff base. That is followed by the reaction of decarboxylation of aminoacid and oxidative deamination. Finally, Carbon dioxide and a C- atom shortened aldehyde formed [39].

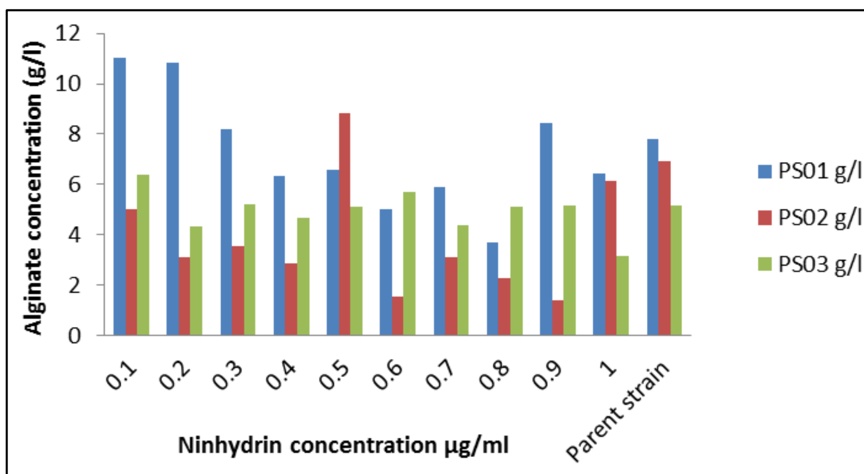


Fig 2: Effect of Ninhydrin on PS01, PS02 and PS03 strains alginate production ability after 96 hours of incubation on a rotary shaker (120 rpm)

BKC mutagenesis

Quaternary ammonium compounds (QAC) like BKC can inhibit the biofilm formation by decreasing the exopolysaccharide production [40]. From the figure 3, it can be observed that the mutation by BKC leads to the inhibition of exopolysaccharide production by PS02-BKC2 strain and PS03-BKC3 strain than the PS02 and PS03. However, PS01-BKC1 showed highest production of alginate biopolymer (11.9 $g.L^{-1}$) than PS01-UV1, PS01-Nin1 and PS01 (Fig. 3) by 1.35 fold, one fold, and 1.56 fold, respectively.

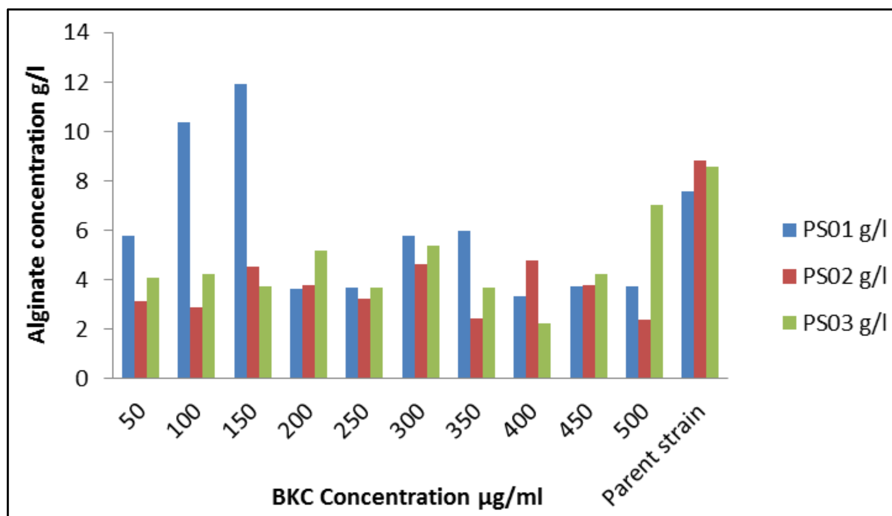


Fig 3: Effect of Benzalkonium chloride on PS01, PS02 and PS03 strains alginate production ability after 96 hours of incubation on a rotary shaker (120 rpm)

Characterization of Extracted alginate biopolymer

FE-SEM

Using field emission scanning electron microscope, we did a morphological analysis of the extracted compounds. Figure 4, figure 5 and figure 6 shows the micrographs of FD-SEM of biopolymer extracted from wild-typestrain PS01, PS02, PS03 identified as *Bacillus Licheniformis, Paenibacillus riograndensis* and *Pseudomonas fluorescence* which is quite identical with the standard sodium alginate.

Furthermore, Figure 7, 8 and 9 demonstrate the EDX spectroscopy analysis with the elements present in the extracted compound.

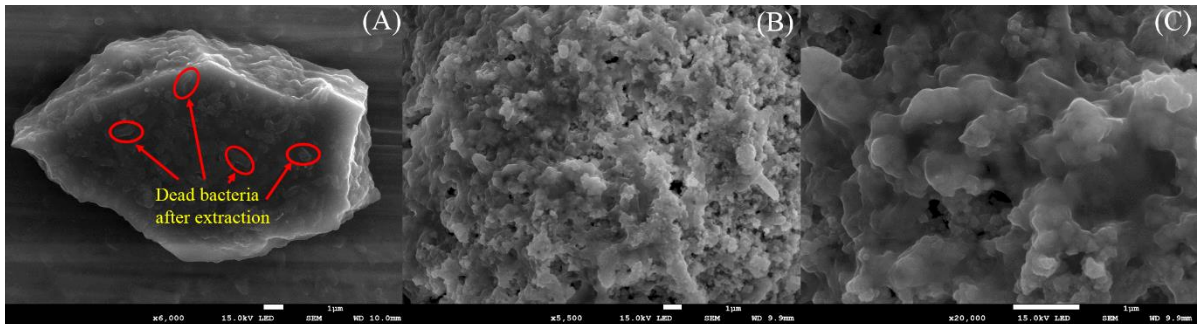


Fig 4: FE-SEM micrographs of sodium alginate extracted from PS01 **(A)** alginate – red circles indicate dead bacteria, **(B)** extracted sodium alginate and **(C)** further zoomed image of extracted alginate.

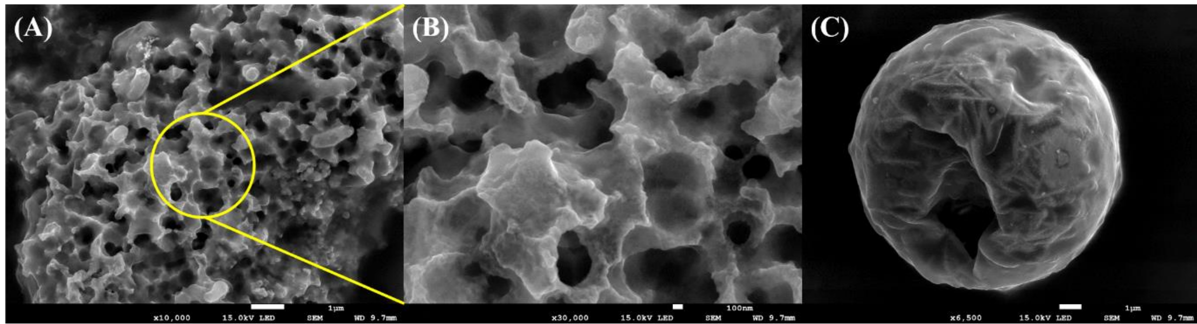


Fig 5: FE-SEM micrographs of sodium alginate extracted from PS02. **(A)** alginate extracted **(B)** further zoomed image of the yellow circle, and **(C)** possible bead-likethe structure of extracted alginate

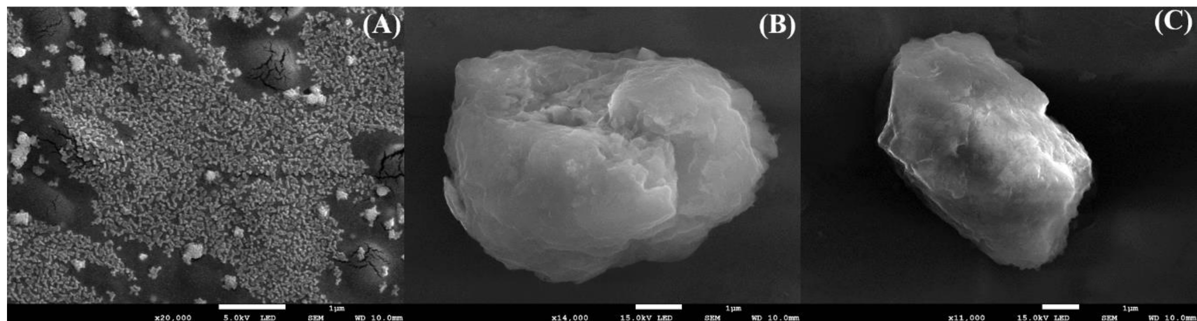
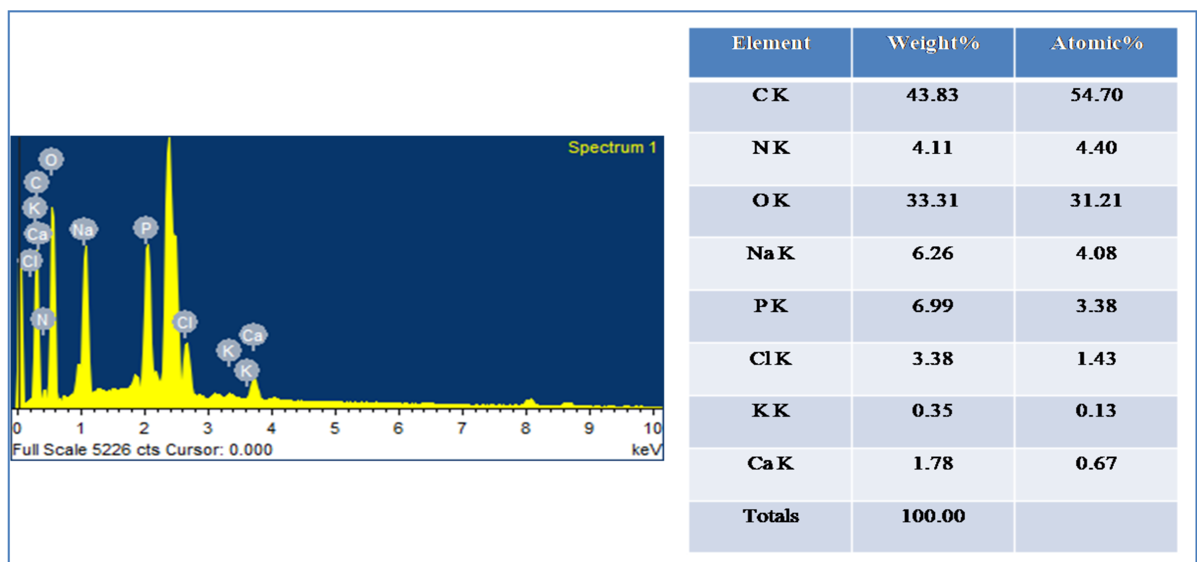
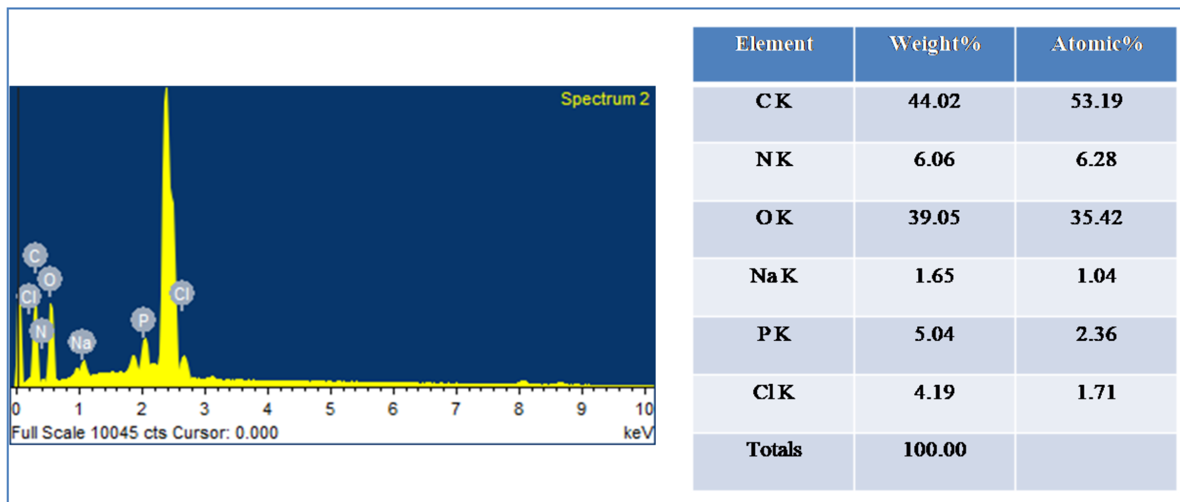


Fig 6: FE-SEM micrographs of sodium alginate extracted from PS03. (A) extracted alginate, (B)micro-sized sodium alginate extracted and (C) different view of extracted sodium alginate.



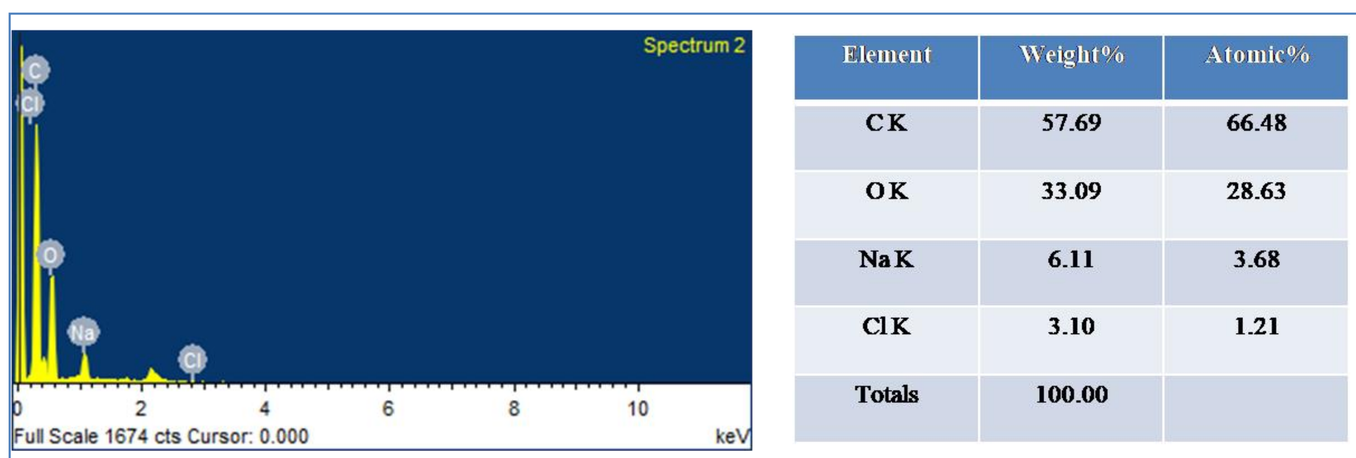
Element	Weight%	Atomic%
C K	43.83	54.70
N K	4.11	4.40
O K	33.31	31.21
Na K	6.26	4.08
P K	6.99	3.38
Cl K	3.38	1.43
K K	0.35	0.13
Ca K	1.78	0.67
Totals	100.00	

Fig 7: Energy Dispersive X-ray spectra (EDX) and elemental analysis of PS01.



Element	Weight%	Atomic%
C K	44.02	53.19
N K	6.06	6.28
O K	39.05	35.42
Na K	1.65	1.04
P K	5.04	2.36
Cl K	4.19	1.71
Totals	100.00	

Fig 8: Energy Dispersive X-ray spectra (EDX) and elemental analysis of PS02.



Element	Weight%	Atomic%
C K	57.69	66.48
O K	33.09	28.63
Na K	6.11	3.68
Cl K	3.10	1.21
Totals	100.00	

Fig 9: Energy Dispersive X-ray spectra (EDX) and elemental analysis of PS03.

FT-IR

IR spectrumconfirms abundance of alginate in extracted alginate fraction from the significant peaks in fig. 10. The significant peaks are relatively comparable with standard alginate. The band at 1633 cm^{-1} can be attributed towards hydrogen bonded O-H stretching vibration [41, 42]. The weak band at 1100 cm^{-1} and 1025 cm^{-1} may be due to C-O stretching vibrations and asymmetric stretching of O-C-O carboxylate from rings, respectively

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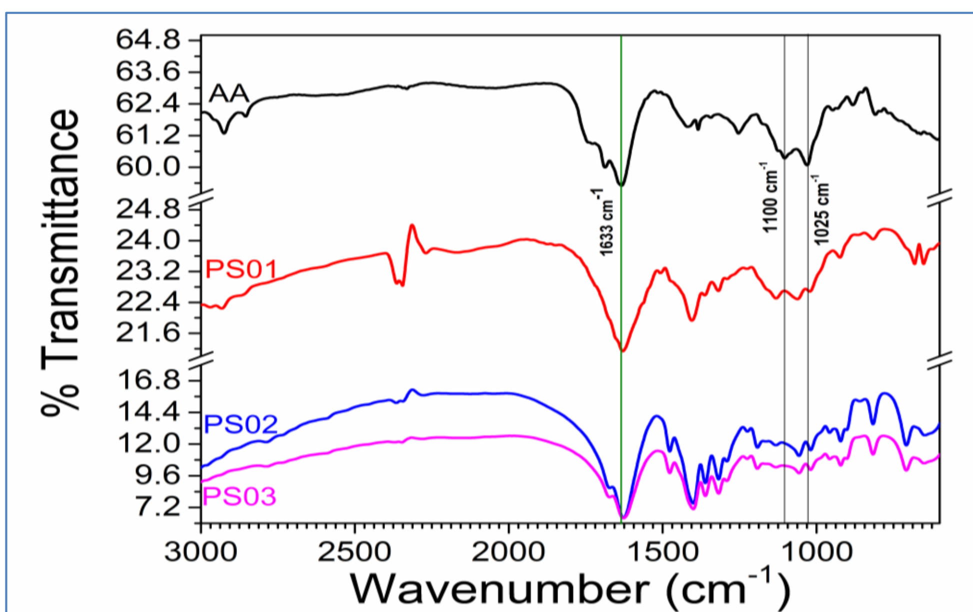

Fig 10: FT-IR spectra of alginate extracted from PS01, PS02,and PS03 strains.

High-pressure liquid chromatography (HPLC)

This chromatography technique confirms the presence of sodium alginate by almost similar retention time of the compound (refer table 1). Figure 10 shows the chromatogram of extracted alginate from PS01, PS02, and PS03 strains.

Table 1: Comparison of standard Sodium alginate with the alginate obtained by the newly isolated strains PS01, PS02 and PS03.

Samples	Retention time (min.)	Area	Height
0.02% Sodium alginate	12.549	32247827	934232
0.08% Sodium alginate	12.556	34460470	966580
0.12% Sodium alginate	12.562	31187858	935436
PS01	12.559	33203568	963522
PS02	12.559	31739080	951471
PS03	12.574	33732547	990798

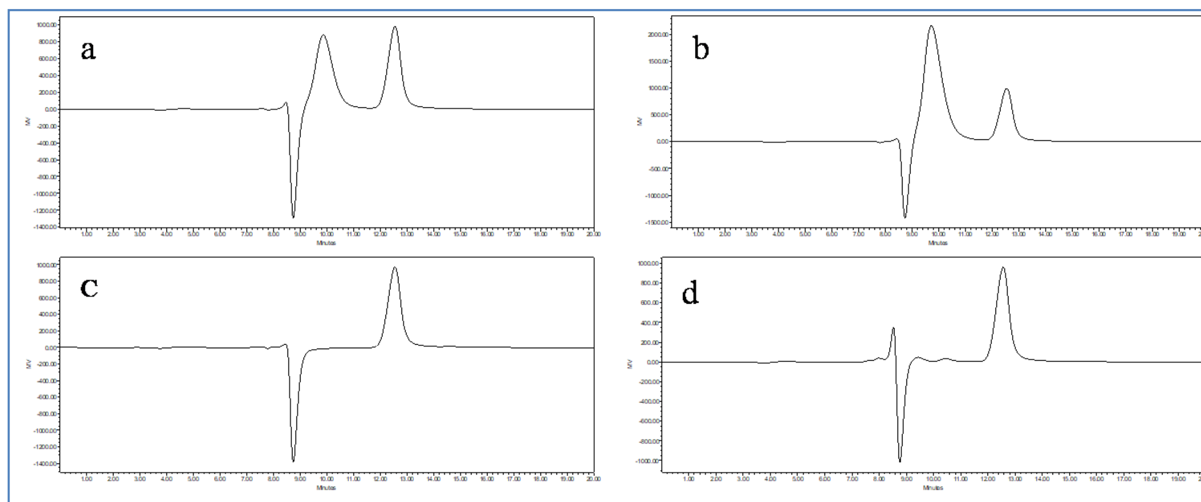

Fig 11: HPLC chromatograms of a. standard sodium alginate b. extracted alginate from ps01 strain c. extracted alginate from the PS02 strain, d. extracted alginate from PS03 strain

Molecular weight determination

Majority of bacterial species secrete high molecular weight polysaccharides when grown under proper culture conditions [7]. Herein, the molecular weight of extracted alginates was determined using GPC. The molecular weight is recorded as 2.3 x 10^3 kDa with the very low polydispersity index 1.54, 2.2 x 10^3 kDa (polydispersity index 1.30) and 2.2 x 10^3 kDa (polydispersity index 1.28).The weight of alginate biopolymer extracted from PS01, PS02, and PS03 strain respectively after the incubation of 96 h.

Conclusion

After creating the mutation in PS01, PS02 and PS03 strains, PS01-Nin1 gives the highest production of alginate 11.04 g.L^{-1} at 0.1 μg/ml ninhydrin concentration. PS02-Nin2 (0.5 μg/ml) and PS02 both gave similar results (8.82 g.L^{-1}), and PS03-UV3 gave 10.66 g.L^{-1} highest alginate concentration after the exposure of 3 min to UV radiation. FE-SEM and EDX results confirmed the presence of C, O, Na, Cl in extracted alginate. HPLC and FTIR results concluded the presence of sodium alginate with O-C-O carboxylate rings and O-H bonds. The molecular weight of the extracted alginate 2.3 x 10^3kDa, 2.2 x 10^3kDa and 2.2 x 10^3kDa of PS01, PS02 and PS03 strain before mutation.

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