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PRODUCTION AND CHARACTERIZATION OF ALGINATE EXTRACTED FROM *PAENIBACILLUS RIOGRANDENSIS*

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ABSTRACT: Alginate is an exopolysaccharide composed of composed of (1-4)-β-D mannuronic acid and its C-5 epimer α-L guluronic acid. In the present study, alginate from bacterial source was tried to produce. The bacteria were isolated from rhizospheric soil of *Momordica charantia* plant, Mehsana, Gujarat, India. Isolation was carried out using selective media for diazotrophic bacteria and biochemical testing. The potential strain was checked for its ability to produce alginate using alginate production assay. Identification of the isolate was carried out by 16s r RNA Sanger sequencing. Molecular characterization was done, andphylogenetic tree was developed. Characterisation of the extracted compound was done by High-pressure liquid chromatography (HPLC), and Fourier-transform infrared spectroscopy (FT-IR). Optimization of the physical parameters (pH, temperature and agitation speed) and chemical parameters (carbon sources, organic and inorganic nitrogen sources) were performed. We have successfully increased the production of alginate biopolymer by optimizing medium from diazotrophic bacteria *Paenibacillus riograndensis*. The findings can aid the production of alginate from bacterial source commercially, as it is of good quality compared to that of algal alginate.

KEYWORDS: Alginate, *Paenibacillusriograndensis,* exopolysaccharide, biopolymer.

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1.INTRODUCTION

Alginate is an extracellular polysaccharide (EPS), which is required for virulence of bacteria by protecting it from different external stimuli such as high and low temperature, pH, the presence of antibiotic [1, 2]. Alginates are linear polysaccharides composed of (1-4)-β-D mannuronic acid and

its C-5 epimer α-L guluronic acid [3] in varying proportions [4]. These residues are arranged in a way that these residues in the molecule are recycled. This molecule is composed of homoblocks M and G andheteroblocks MG [5]. Alginate was first named algic acid or algin,and only a few of its properties were known [6, 7]. Alginate contains negative charge on it, which enables to form higher viscosity solutions. In the absence of divalent cations, forms viscous solution. While in the presence of divalent or trivalent cations, forms the gel-like structure [8]. Alginate has a common molecular formula $(C_6H_8O_6)_n$, with a molecular mass varied between 10,000 and 6,00,000 [8]. Alginate occurs as structural components in marine brown algae (Phaeophyceae) as well as exopolysaccharide (capsular) in some bacteria [9]. Nowadays, commercial alginates are extracted from brown seaweeds such as *Laminaria digitata, Mycrocystis pyrifera* and *Laminaria hyperborea* [4]. Several nitrogen-fixing bacteria such as *Azotobacter vinelandii* [10, 11] and opportunistic pathogen *Pseudomonas aeruginosa* also produces alginate [12]. Although seaweeds are the significant source of alginate, however, the bacterial alginate is considered of good quality than of the algal alginate. The bacterial alginate has the higher selling price due to its better qualitative properties [13]. Alginate has widespread industrial applications. About 30,000 metric tons of sodium alginate per year used as an emulsifier in foods, paper making industry, textile, jellifying agents, thickening agents and stabilizers [14]. More than half of the total worldwide alginate produced is used in the industries such as creams and cake mixtures, icy custards, ice cream, as well as to keep the contents in suspension in fruit juices [4]. Alginate prevents crystallization and shrinkage in ice cream which results in the homogenous product. Sodium alginate or ester of propylene glycol alginate used in salad dressing as a stabilizer by preventing phase separation. With the same principle, it is used in mayonnaise emulsion phase water/oil [15]. Alginate has been used in pharmaceutical industries as an anti-inflammatory agent, wound healing, radioactive suppressive agent [16, 17]. Different pathogenic and non-pathogenic spp. has been recognized to produce alginate biopolymer. Alginate from *Pseudomonas sp.* and *Azotobacter sp.* are widely used in industries [8]. Therefore researchers are more interested in alginate production by different other species. A mutant c-14 of *Azotobacter vinelandii* NCIB 9068 was found to produce alginate, and its properties comparison with algal alginates was investigated [18]. Bacterial alginate showed more pseudoplasticity than of algal, whereas all the other parameters like gel formation capacity, thermostability, effect of pH, temperature and NaCl on viscosity were significantly similar. Alginate production using glucose as the carbon source by *Azotobacter vinelandii* DSM 576 was optimized with respect of agitation speed, C/N ratio, sodium phosphate and acetate in a buffered medium, glucose concentration and fermentation temperature. [19]. Parente et al. concluded that the higher concentration of alginate yield was obtained when dissolved oxygen was not controlled. They further confirmed that high concentrationswere obtained after the growth period (end of stationary phase) [20]. Clementi et al. studied the detailed procedure for alginate production in shake flask culture and laboratory scale by

Azotobacter vinelandii. [10]. Here, in present study production and characterization alginate from diazotrophic bacteria such as *Paenibacillus sp.* has been done. Then et al. studied alginate production in semi-industrial scale 16L and 150L stirred tank bioreactor. The maximum alginate production achieved in this study was 18.6 g/l using fed-batch cultivation strategy in 150 L stirred tank bioreactor [8].Azotobactervinelandii A3 strain was studied for its alginate production ability [3]. When bacterial alginate was added to Cu^{2+} and Zn^{2+} ions, it forms gel structures. A recent study demonstrated that the psychrotolerant *Pseudomonas mandelii* was found to overproduce alginate [21]. A novel 6A1 strain of *A. Mandelii* isolated from antarctice increases biofilm formation due to alginate overproduction at low temperatures. This might be the result of down regulation of MucA (alginate operon repressor). Bacterial alginate production process is costly due to the carbon source used. Molasses, a byproduct of sugar manufacturing can be used as a cheap carbon source. *Azotobacter vinelandii* ATCC 9046 strain was inoculated into the modified Burk's medium (Changing sugar source) for alginate production. To make the bacterial alginate cost-effective use of molasses resulted in high yield than maltose. Alginate-clinoptilolite beads were efficiently adsorbed copper up to 131.6 mg Cu^{2+}/g adsorbent at pH 4.5 [22].In the present study, a novel diazotrophic strain *Paenibacillus riograndensis* PS02 was found to produce alginate biopolymer. Optimization of the common parameters was done to maximize the alginate production by the strain. The isolated strain is nonpathogenic. Characterization of alginate was done morphologically by scanning electron microscopy, and detailed elemental analysis was done by Scanning Electron microscopy (SEM) and Energy dispersive X (EDX) ray spectroscopy.Future work can be done on characterization (molecular weight), purity and finding the similarity with the algal alginate.

2. MATERIALS AND METHODS

Soil samples were collected from 17 different agricultural areas of North Gujarat Region during winter, summer and monsoon seasons to check the ecological diversity of nitrogen-fixing bacteria to check seasonal variations.

Collection of the sample

Samples were collected from the rhizosphere of the plant*Momordica charantia*from the mehsana district (Latitude 23°37'59.41"N; Longitude 72°23'38.89"E). The soil was taken from the depth of 6 to 15cm, after discarding the 5cm uppermost layer. The sample was then stored in the refrigerator for immediate use. However, for long-term usage, samples were stored at 4°C.

Isolation of diazotrophic bacteria

Different selective media for the isolation of nitrogen fixing bacteria was used throughout the study such as Jensen's media (20g sucrose, 1.0g dipotassium phosphate, 0.5g magnesium sulphate, 0.5g sodium chloride, 0.1g ferrous sulphate, 0.005g sodium molybdate, 2.0g calcium carbonate, 15g agar) [23], Azotobacter media (20g mannitol, 0.2g K_2HPO_4, 0.2g $MgSO_4$, 0.2g NaCl, 0.1g K_2SO_4, 5.0g $CaCO_3$, 15.0g Agar) and Burk's nitrogen free medium (10g glucose, 0.41g KH_2PO_4, 0.52g

Na_2SO_4, 0.2g $CaCl_2$, 0.1g $MgSO_4.7H_2O$, 0.005g $FeSO_4.7H_2O$, 0.0025g, $Na_2MoO_4.2H_2O$, 1.8g agar) (all per litre distilled water) [24]. The pH of the medium was adjusted to 7 +/- 0.1 before autoclaving at 121°C for 15 minutes. Phenotypic characterization of bacterial isolates was carried out by methods described in the Bergey's manual of systematic bacteriology [25]. The isolates were characterizedby the following traits: color, the margin of the colony, opacity and texture, surface, color-pigment production. Grams reaction was performed as per the standard protocol. Plate assays were performed to check the presence of caseinase, amylase, lipase enzymes. Carbon source utilization was tested for sucrose, glucose, arabinose, maltose, mannitol, galactose, lactose, fructose, xylose, and dextrose.

Molecular characterization

DNA isolation was carried out using DNA blood minikit by Qiagen (as per the manufacturer protocol). Extracted genomic DNA was quantified using nanodrop lite 100 by thermo scientific instrument. The amplification of 16s rRNA gene portions from the bacterial sample was performed in PCR (Applied biosystem verity- 96 well gradient thermal cycler) by using the forward primer AGAGTTTGATCMTGGCTCAG(20) with the oligo name 27F and the reverse primer TACGGYTACCTTGTTACGACTT(22) with the oligo name 1492R. Amplification performed in 50µl reaction volume containing DNA template 4µl, master mix 24µl (Takara bioscientific), F-primer 2µl, R-primer 2µl and water 18µl.The thermal cycling was performed as follows: initial denaturation at 95°C for five mins, followed by 35 cycles of 94°C for 30 seconds, 58°C for 1 minute, 72°C for 45 seconds and a final extension at 72°C for 5 minutes. The PCR products were analyzed by running 5-10 µl aliquots of the reaction mixture in 1% agarose ethidium bromide. The obtained 16s rRNA gene was sequenced by the applied biosystems company by sanger sequencing method using the same primer. The instrument used for sanger sequencing was 3730xl/3730XLPC-19137-017 utilizing the sequence scanner software 2 v2.0. Sequence analysis was performed by alignment of the partial 16s rRNA gene sequences to those from the GenBank databank, using the program BLAST (NCBI BLAST^R homepage). The nucleotide sequence of the 16s rRNA gene segments determined in this study has been deposited in GenBank (NCBI) under the accession number MG432472. A preliminary phylogenetic analysis was created using the NCBI gene databank.

***nif* Gene gel electrophoresis**

As this isolated PS02 strain is recently discovered to fix nitrogen in the plant rhizosphere. Therefore *nifH* gene amplification was done to confirm. Selected primers PolF and PolR (TGCGAYCCSAARGCBGACTC and ATSGCCATCATYTCRCCGGA, respectively, [26] were used to amplify a very well conserved 360-bp region of the nifH gene [27]. The reaction specification and PCR conditions were same as used for DNA amplification for 16s rRNA gene sequencing. The gel electrophoresis was performed using commercial DNA ladder, and the PS02 strain was compared with it. DNA bands were visualized using UV illuminator.

Alginate extraction procedure

The PS02 strain was inoculated in alginate production (AP3) medium 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 [28]. Erlenmeyer flask containing 50 ml of alginate production medium was inoculated and incubated at 28°C and 120 rpm for 96 hours.

Cell biomass separation and alginate precipitation

Separation of cell biomass and alginate extraction from culture was achieved by Clementi, 1997 method [10]. 5 ml of culture broth was taken into the pre-weighed centrifuge tube. 0.2 ml of 0.5 M Na_2EDTA was, and 0.1 ml of 5 M NaCl was added to remove the cell-bound alginate into the culture. The culture was centrifuged at 6000 rpm for 30 mins at 4°C to separate the biomass. The supernatantwas collected in different pre-weighed tubes for alginate precipitation. Pellet was washed, dried and weighed for cell biomass. Twice the volume of ice coldisopropyl alcohol (IPA) was added to the supernatant to precipitate alginate. The mixture was centrifuged at 6000 rpm for 45 mins to pellet out precipitated alginate. The supernatant was carefully removed,and the pellet was dried at 105°C for 24 hours.

Determination of physical properties of alginate

The following properties of alginate were observed and recorded: color, a feature of alginate and its different solubility (ethanol, methanol, isopropanol, and water).

Morphological analysis by SEM and elemental analysis

Surface morphology and Energy Dispersive X-ray (EDX) spectroscopy(elemental analysis) were done by field emission scanning electron microscope (FE-SEM) JFM 7100 F (oxford incorporation). The 0.01 wt% solution of the samplewas prepared in miliQ water (10µl). The solution was carefully poured onto copper stub and allowed to dry in an oven at 60°C and gold coated with sputter coater.

Characterization of alginate by Fourier-transform infrared spectroscopy (FT-IR) and High-Pressure Liquid Chromatography (HPLC)

FT-IR measurements were carried out at room temperature using Nicolet 6700 FT-IR spectrometer. 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}.

Shimadzu Prominence HPLC system was used for the analysis equipped with an RI detector. Enable C18H 5µm 150 mm x 4.6 mm column used, and 5mM $H_2SO_4$was used as a mobile phase [29]. The flow rate of the mobile phase 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).

Optimization of the medium parameter in a shake flask

To check the effect of different physical and chemical parameters on alginate production, the AP3 medium was used. Only one parameterwas checked at the time, and all the other parameters were

kept unchanged. All of the determinations were analyzed according to the alginate production method described above.

Physical parameters

Temperature

To analyze the effect of different temperatures on alginate production, the AP3 medium was inoculated and kept at temperature ranges between 20°C to 45°C with the difference of 5°C were analyzed.

pH

pH of the AP3 medium was adjusted in the range from 3 to 11 using the 1N HCl or 1N NaOH by the difference of 1 to check its consequence on alginate production by PS02 strain.

Agitation speed

For determining the optimum speed and effect of aeration on alginate exopolysaccharide production, the flasks were kept at a different speed such as static, 50, 100, 150 rpm.

Chemical parameters

Carbon source

To study the effect of carbon source on alginate production from the medium AP3 sucrose carbon source was replaced in 2% concentration by each of the following sugars. Different carbon source such as glucose, lactose, mannitol, maltose, sucrose and fructose [7] were analyzed.

Nitrogen source

To study the effect of organic and inorganic nitrogen source on alginate production from the medium AP3 yeast extract and $(NH_4)_2SO_4$ source was replaced in O.6% and 0.06% concentration by each of the following nitrogen sources, respectively. Organic nitrogen sources such as beef extract, casein, corn steep liquor, malt extract, peptone, yeast extract [7] and soybean meal. Inorganic nitrogen sources such as $NaNO_3$ and $(NH_4)_2PO_4$ were checked.

3. RESULTS AND DISCUSSION

Isolation and purification of potential isolates

A strain, PS02, was isolated from the rhizospheric soil of the *Momordica charantia* plant, Mehsana, Gujarat. Large numbers of nitrogen-fixing isolates were obtained using the Jensen's medium. Fig. 1a shows the growth of PS02 on Jensen's media produces gummy, mucoid, circular, even margin, transparent, convex colonies after incubation of 48-72 hrs at 33°C. PS02 isolate is a gram-positive short bacilli (Fig. 1b), aerobic and motile. The isolated PS02 strain produces spore (refer Fig. 1c) and capsule. PS02 grows at the optimum temperature of 33°C with optimum pH 7 to 7.5. This strain can ferment different carbohydrate like sucrose, dextrose,and fructose without gas production and cannot ferment glucose, arabinose, maltose, mannitol, galactose, lactose, xylose. The catalase test was found to be positive, whereas gelatine test negative.

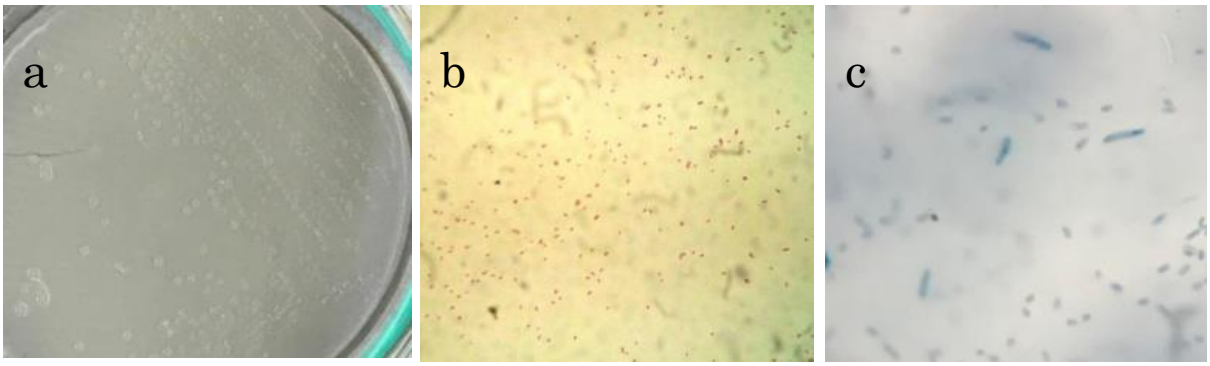

Fig.1 Primary identification of PS02 strain a). growth of PS02 on jensen's media after 72 hours, b) gram staining of PS02, c) spore staining of PS02

Sequencing and phylogeny of 16S rRNA

Isolated DNA was quantified using nanodrop lite 100 instrument which shows 1.47 ratio with the concentration of 26.2 ng/µl. Comparison of 16s rRNA gene sequence of strain PSO2 (GenBank accession number MG432472) with the sequence held in the NCBI gene bank database revealed that this bacterium clustered with species of the genus *Paenibacillus*(Fig. 2). A phylogenetic tree was constructed based on the 16s rRNA gene sequences was shown in Figure 2.

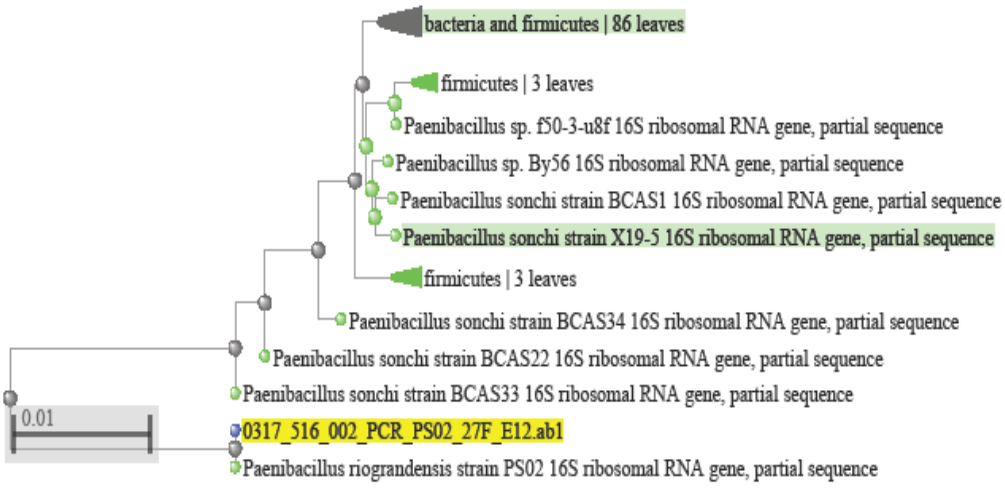

Fig. 2 Phylogenetic tree of PS02 strain

***nif* gene gel electrophoresis**

Many species of bacilli can be detected in the soils and rhizosphere [30]. Nitrogen-fixing ability of the Bacillus species was first demonstrated by Ding et al., 2005. Though, Xie et al. (2003) has isolated many *Bacillus species* (*B. licheniformis, B. cereus, B. megaterium, B. azoformans and B. subtilis*) from the rhizosphere of rice plants and studied it nitrogen-fixing ability [31]. A significant number of *Bacillus species* from rhizosphere and soil environment was detected in this study was belonging to *Paenibacillus species*. Seldin et al. (1998) and Rosado et al. (1998) reported nitrogen-fixing *Paenibacillus durus* and *P. polymyxa* were the most commonly isolated species [32, 33]. Although here in this study, these two species were not found may be due to the differences in the physical properties and temperature. The isolated novel strain was checked for the presence of

Nifgene using gel electrophoresis. Gel electrophoresis result (Fig. 3) indicates the band at 360bp region by comparing with the ladder DNA. The *nif*gene-specific primer used was of 360 bp, which confirms that the isolated novel *Paenibacillus* strain contains *nif* gene.

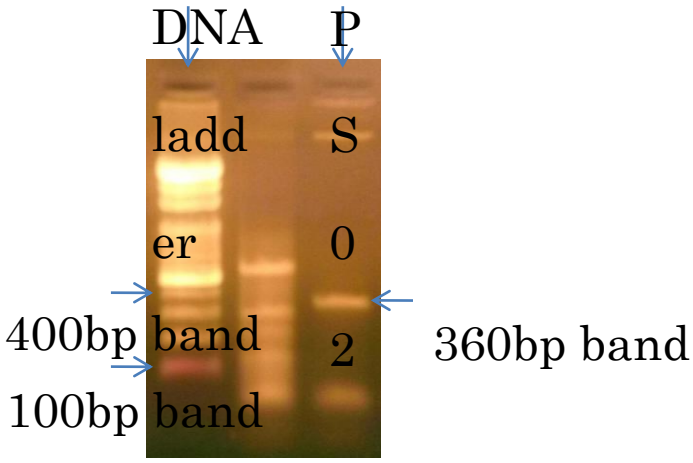

Fig. 3 *nif* gene gel electrophoresis of the PS02 strain PCR product

Screening Fermentation medium

Different components of the medium play vital role in the production of alginate and growth of microorganisms. Medium components fulfill the nutritional requirement for the optimal growth and polymer production by microorganisms [34]. Four different media were used for the alginate production [35, 8, 22, 3] (fig. 4). It can be concluded from the fig. 4 that out of all, AP3 medium gives the maximum results 5.24 g/l. This might be due to the presence of sucrose as a sole source of carbon and yeast extract as a nitrogen source [36].

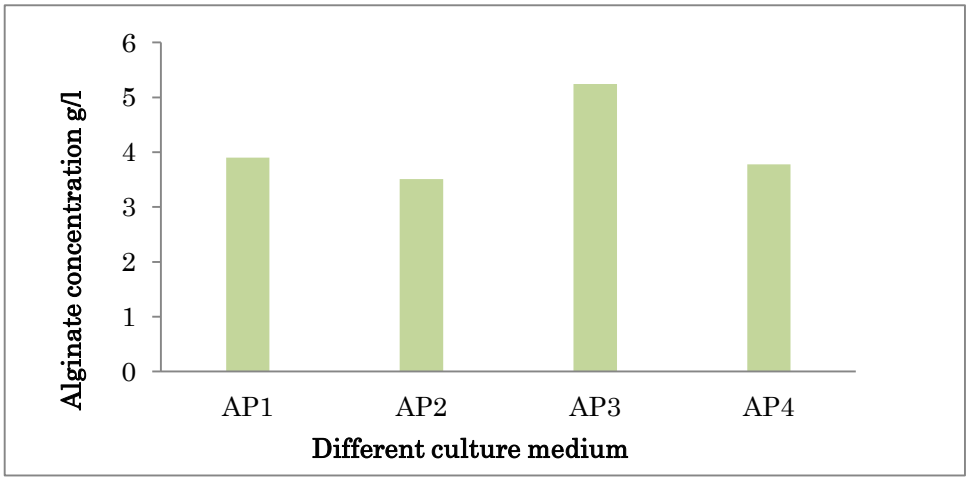

Fig. 4 Medium screening for maximum alginate production from *Paenibacillus riograndensis*

Morphological and elemental analysis of extracted alginate compound

SEM micrographs demonstrate (Fig. 5) that the extracted alginate compound and standard alginate compound shows approximately similar morphological structure. Further, EDX spectroscopy analysis showed almost identical elements in standard sodium alginate (Himedia) and extracted compound. Hence, confirmed from table 1 and fig. 6 that the extractedcompound was alginate.

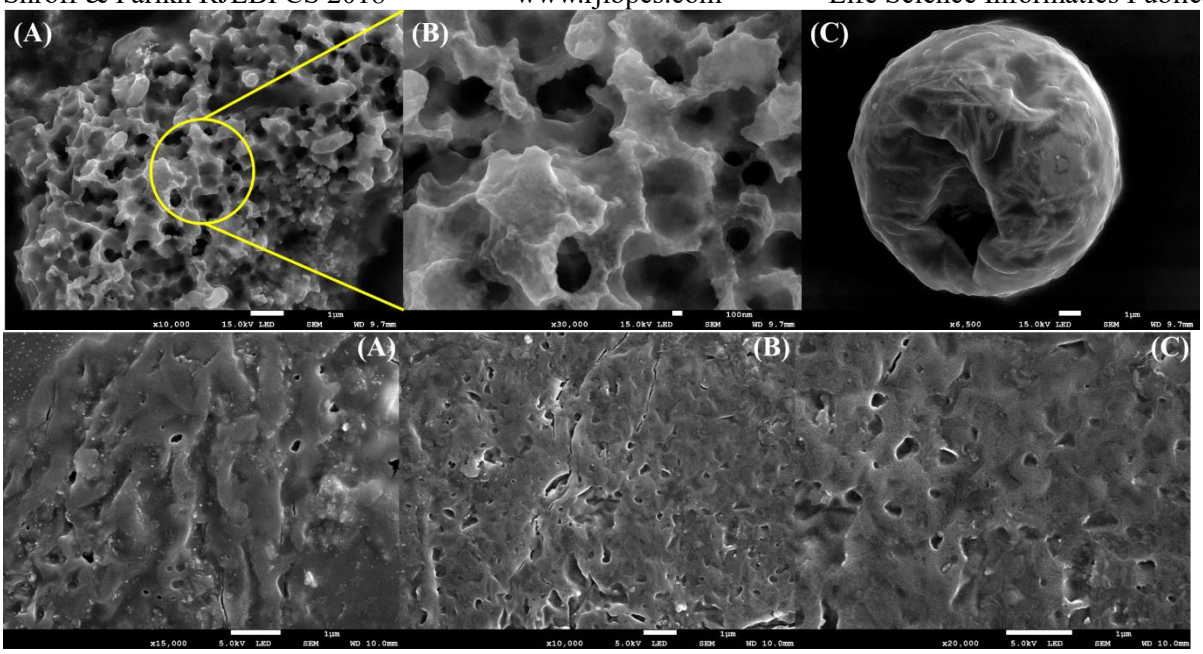

Fig. 5 FE-SEM micrographs (top) sodium alginate extracted from PS02 strain (A) alginate extracted (B) further zoomed image of the yellow circle and (C) possible bead-like structure of extracted alginate, (bottom) standard sodium alginate (A, B) different angles of alginate and (C) further zoomed image of B

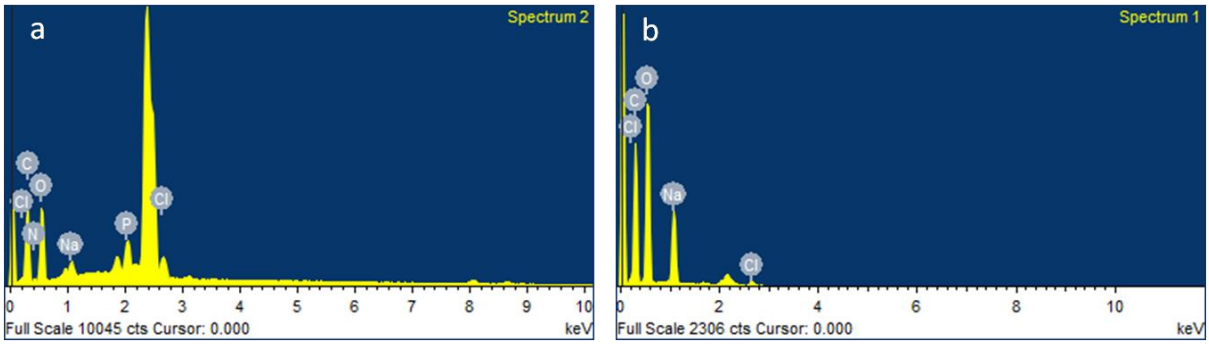

Fig. 6 Comparison of the Energy dispersive x-ray spectroscopy analysis (EDX) a) extracted compound from the PS02 strain, b) standard sodium alginate.

Table 1: Comparison of elemental analysis by EDX (left) extractedcompound (right) standard sodium alginate

Element	Weight%	Atomic%
CK	44.02	53.19
NK	6.06	6.28
OK	39.05	35.42
NaK	1.65	1.04
PK	5.04	2.36
ClK	4.19	1.71
Totals	100.00	

Element	Weight%	Atomic%
CK	34.60	44.63
OK	43.69	42.32
NaK	15.04	10.14
ClK	6.66	2.91
Totals	100.00	

Characterization of extracted alginate by IR and HPLC

IR spectrum confirms high abundance of sodium alginate in extracted alginate fraction. As we can observe significant peaks in fig. 7 for alginate confirmation. The significantpeaks are quite comparable with standard sodium alginate. The band at 3424 cm^{-1} can be attributed towards hydrogen bonded O-H stretching vibration, the band at 1615 cm^{-1} can be attributed towards asymmetric stretching of O-C-O carboxylate. The band at 1415 cm^{-1} may be attributed to C-OH deformation vibration with the contribution of O-C-O symmetric stretching of the carboxylate group [37, 38]. The weak band at 1125 cm^{-1} may be due to C-O stretching vibrations from rings.

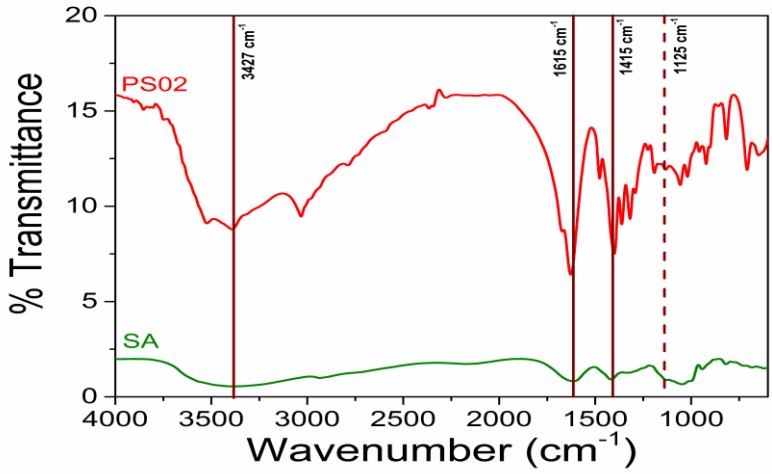

Fig. 7 Characterization of extracted alginate by FT-IR spectroscopy

HPLC analysis of extracted alginate was carried out for confirmation of sodium alginate in the extract. As from Table. 2, the retention time of extracted alginate is similar to that of standard sodium alginate. HPLC chromatogram showed in the Fig. 7 confirms that the extracted compound was sodium alginate.

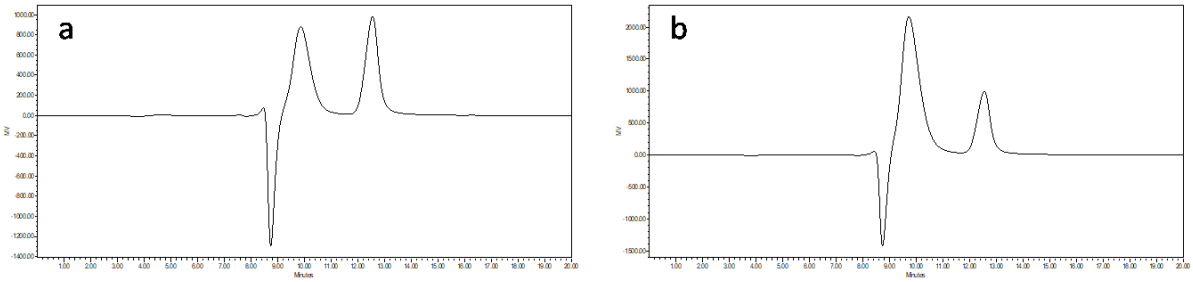

Fig. 8 HPLC Chromatogram a) Standard 0.02% sodium alginate, b) PS02 extracted compound

Table 2: HPLC results of a compound extracted by PS02 strain and standard sodium alginate (Himedia)

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
PS02	12.559	31739080	951471

Optimization of AP3 medium for maximum alginate production

Biopolymer accumulation by different bacteria has been reported to be affected by their nutritional, physical and growth requirements such as carbon sources, nitrogen sources, pH, temperature and agitation speed. Therefore all these parameters were optimized for obtaining maximum production of alginate.

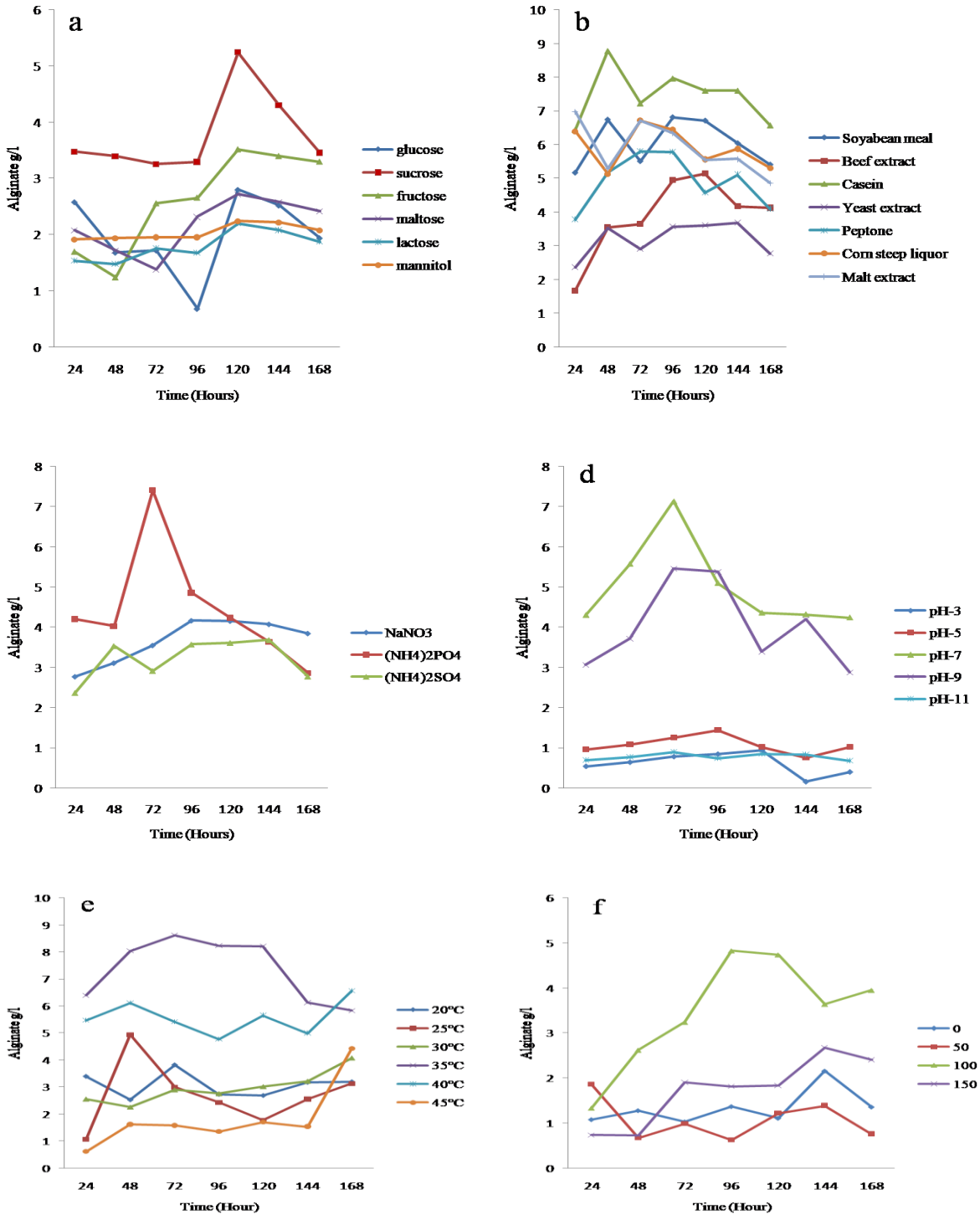

Fig.9 Effect of different chemical and physical parameters on the production of alginate biopolymer by *P.riograndensis*at the internal of 24 hours for 168 hours. a) Carbon sources, b) Organic nitrogen sources, c) inorganic nitrogen sources, d) pH, e) temperature, f) agitation speed

Carbon source

Carbon source has a dual effect as a building block and the energy requirements for the organism. Readily absorbable and suitable sugars can be the best choice for the bacterial culture to obtain good production [29, 39]. In the present study, different carbon sources (fig.9a) Sucrose proved to be the best sole source of carbon. When sucrose is used as a carbon source in the alginate production medium *P.riograndensis* gives the highest amount of alginate 5.24 g/l. The reason for this might be that the sucrose is readily metabolized and readily employed by the bacteria [30]. Other carbon sources might be slowly metabolized by the bacteria.

Nitrogen source

Different organic and inorganic nitrogen sources were used for optimization of the AP3 medium. It can be concluded from the fig. 9b that when casein is used as an organic nitrogen source with $(NH4)_2SO_4$ it gives the highest production of 8.79 g/L alginate. While $(NH_4)_2PO_4$ used as the inorganic nitrogen source with yeast extract as an organic source gives the highest production of 7.41 g/L alginate (fig. 9c).

pH

pH of the fermentation medium is the crucial parameter for both alginate production and bacterial growth. In the present study, two acidic, two basic and one neutral pH was taken (Fig.9d). As per the chart, it is confirmed that the neutral pH 7 gives the maximum alginate production of 7.14 g/L whereas; pH 5, 3 and 11 doesn't provide any quantifiable amount of alginate.

Temperature

The flasks were incubated at different temperatures (20, 25, 30, 35, 40, 45) for 168 hours. The excellent alginate production obtained at 35°C temperature and after 72 hours (8.62 g/l) (fig. 9e).This bacterial strain is mesophilic, so bacterial metabolic activities get disturbed at high temperatures. The bacteria produce alginate at the end of trophophase and start of idiophase. This bacterial strain can produce cysts in the unfavorable conditions and alginate is the main component of cyst [40, 41]. After nutrient limiting conditions takes place, it triggers the bacteria to use the produced alginate which leads to the decrease in alginate production.

Agitation Speed

A proper agitation speed is essential for adequate mixing of the medium components and to provide appropriate air supply. Aeration is also a very major parameter responsible for the appropriate growth of the bacteria. In the current study, the maximum amount of alginate produced with the agitation speed of 100 rpm (4.83 g/L) (fig. 9f). Further increase in agitation speed decreases the alginate production due to breakage of the bacterial cells. While low agitation speed decrease the alginate production due to the not proper mixing of the medium components so that cannot be made available to the microorganisms.

Alginate production by optimized media parameters

As per the study, *Paenibacillus spp* gives the highestproduction of alginate with the sucrose as a carbon source, casein as an organic nitrogen source and $(NH_4)_2PO_4$ as an inorganic nitrogen source. Though, optimized parameter medium do not give the highest production of alginate due to casein is insoluble in water thus leads to the false positive results. Therefore it can be concluded that the highest alginate production can be achieved with the same AP3 medium at 35°C.

4. CONCLUSION

In the present study, newly recognizednitrogen-fixing diazotrophic spp. *Paenibacillus riograndensis* strain isolated from the rhizosphere of the *Momordica charantia* plant of North Gujarat region of latitude 23°37'59.41"N and longitude 72°23'38.89"E of Mehsana district. The isolated strain investigated to produce biopolymer alginate. Molecular characterization by 16s rRNA sanger sequencing and phylogenetic analysis revealed that the isolated PS02 strain belongs to *Paenibacillus spp*. The sequence was submitted to the GenBank with the accession number MG432472.Morphology study of the alginate by SEM and EDX shows almost similarity in structure and elements present. Characterization of extracted alginate by HPLC and FT-IR results also confirmed that the extracted compound is alginate. Four different alginate production media AP1, AP2, AP3,and AP4 were checked for the highest production of alginate. It can be concluded from the figure 4 that out of this AP3 gives the best results. The AP3 medium was used to check the effect of different physical and chemical parameters on alginate production. Optimization study concludes that PS02 strain gives the highestproduction of alginate at 35°C and keeping the agitation speed of 120 rpm (figure 9). This significantly increased the production of alginate from 5.24g/l to 8.62 g/l. Further studies on purification and characterization of alginate parameters can be done.

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CONFLICT OF INTEREST

There is no conflict of interest in the present work.

REFERENCES

1. Khanafari A &Sepahei AA. Alginate biopolymer production by *Azotobacter chroococcum* from whey degradation. International Journal of Environmental Science & Technology, 2007; 4: 427-432.
2. Vu B, Chen M, Crawford RJ & Ivanova EP, Bacterial extracellular polysaccharides involved in biofilm formation. Molecules, 2009; 14: 2535-2554.
3. Auhim HS & Hassan SS, Production and characterization of alginate from *Azotobacter*

vinellandii A3. Int J Adv Pharm Biol Chem, 2013; 2: 507-512.

4. Saude N, Chèze-Lange H, Beunard D, Dhulster P, Guillochon D, Cazé A, Morcellet M & Junter G, Alginate production by *Azotobacter vinelandii* in a membrane bioreactor. Process Biochemistry, 2002; 38: 273-278.
5. Smidsrød O, Solution properties of alginate. Carbohydr Res, 1970; 13: 359-372.
6. Díaz-Barrera A, Silva P, Avalos R & Acevedo F, Alginate molecular mass produced by *Azotobactervinelandii* in response to changes of the O2 transfer rate in chemostat cultures. Biotechnol Lett, 2009; 31: 825-829.
7. Butt ZA & Qadeer M, Alginate production by a mutant strain of *Azotobacter vinelandii* using shake Flask fermentation. Pak J Bot, 2011; 43: 1053-1067.
8. Then C, Othman Z, Mustapha WW, Sarmidi M, Aziz R & El Enshasy H, Production of alginate by *Azotobactervinelandii* in semi-industrial scale using batch and fed-batch cultivation systems. Journal of Advanced Scientific Research, 2012; 3: 45-50.
9. Draget KI, Skjåk-Bræk G & Stokke BT, Similarities and differences between alginic acid gels and ionically crosslinked alginate gels. Food Hydrocoll, 2006; 20: 170-175.
10. Clementi F, Alginate production by *Azotobacter vinelandii*. Crit Rev Biotechnol, 1997; 17: 327-361.
11. Gorin P & Spencer J, Exocellular alginic acid from *Azotobacter vinelandii*. Canadian Journal of chemistry, 1966; 44: 993-998.
12. Evans LR & Linker A, Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J Bacteriol 1973; 116: 915-924.
13. Ertesvag H, Erlien F, Skjak-Braek G, Rehm BH & Valla S, Biochemical properties and substrate specificities of a recombinantly produced *Azotobacter vinelandii* alginate lyase. J Bacteriol, 1998; 180: 3779-3784.
14. Brownlee I, Allen A, Pearson J, Dettmar P, Havler M, Atherton M & Onsøyen E, Alginate as a source of dietary fiber. Crit Rev Food Sci Nutr, 2005; 45: 497-510.
15. da Silva AN & Garcia-Cruz CH, Biopolymers by *Azotobacter vinelandii*. In: Anonymous Biopolymers. InTech 2010
16. Kammerlander G &Eberlein T, An assessment of the wound healing properties of Algisite M dressings. Nurs Times, 2003; 99: 54-56.
17. Mirshafiey A, Rehm B, Sotoude M, Razavi A, Abhari RS & Borzooy Z, Therapeutic approach by a novel designed anti-inflammatory drug, M2000, in experimental immune complex glomerulonephritis. Immuno pharmacol Immuno toxicol, 2007; 29: 49-61.
18. Chen WP, Chen JY, Chang SC & Su CL, Bacterial Alginate Produced by a Mutant of *Azotobacter vinelandii*. Appl Environ Microbiol, 1985; 49: 543-546.
19. Clementi F, Fantozzi P, Mancini F & Moresi M, Optimal conditions for alginate production by

Azotobacter vinelandii. Enzyme MicrobTechnol, 1995; 17: 983-988.

20. Parente E, Crudele M, Aquino M & Clementi F, Alginate production by *Azotobacter vinelandii*DSM576 in batch fermentation. Journal of Industrial Microbiology and Biotechnology, 1998; 20: 171-176.
21. Vasquez-Ponce F, Higuera-Llantén S, Pavlov MS, Ramírez-Orellana R, Marshall SH & Olivares-Pacheco J, Alginate overproduction and biofilm formation by psychrotolerant Pseudomonas mandelii depend on temperature in Antarctic marine sediments. EJB, 2017; 28: 27-34.
22. Kıvılcımdan Moral Ç & Yıldız M, Alginate production from alternative carbon sources and use of polymer based adsorbent in heavy metal removal. International Journal of Polymer Science, 2016; 2016: 1-8.
23. Kumar V &Narula N, Solubilization of inorganic phosphates and growth emergence of wheat as affected by Azotobacterchroococcum mutants. Biol Fertility Soils, 1999; 28: 301-305.
24. Laskar F & Sharma G, Isolation and Characterisation of Diazotrophic Bacteria from Rhizosphere of different Rice Cultivars of South Assam, India. Current World Environment, 2013; 8: 157-163.
25. Holt JG, Krieg NR, Sneath Pa, Staley JT & Williams ST, Bergey's Manual of determinate bacteriology (1994).
26. Poly F, Monrozier LJ & Bally R, Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Res Microbiol, 2001; 152: 95-103.
27. Beneduzi A, Peres D, Vargas LK, Bodanese-Zanettini MH & Passaglia LMP, Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil. Applied Soil Ecology, 2008; 39: 311-320.
28. Nosrati R, Owlia P, Saderi H, Olamaee M, Rasooli I & Akhavian TA, Correlation between nitrogen fixation rate and alginate productivity of an indigenous Azotobacter vinelandii from Iran. Iran J Microbiol, 2012; 4: 153-159.
29. Kim TH, Choi WI, Kim YS & Oh KK, A novel alginate quantification method using high-performance liquid chromatography (HPLC) for pretreatment of *Saccharina japonica*. J Appl Phycol, 2015; 27: 511-518.
30. Ding Y, Wang J, Liu Y & Chen S, Isolation and identification of nitrogen- fixing bacilli from plant rhizospheres in Beijing region. J Appl Microbiol, 2005; 99: 1271-1281.
31. Xie GH, Cai MY, Tao GC & Steinberger Y, Cultivable heterotrophic N 2-fixing bacterial diversity in rice fields in the Yangtze River Plain. Biol Fertility Soils, 2003; 37: 29-38.
32. Seldin L, Rosado AS, da Cruz DW, Nobrega A, van Elsas JD & Paiva E, Comparison of *Paenibacillus azotofixans* strains isolated from rhizoplane, rhizosphere, and non-root-

associated soil from maize planted in two different brazilian soils. Appl Environ Microbiol, 1998; 64: 3860-3868.

33. Rosado A, De Azevedo F, Da Cruz D, Van Elsas J & Seldin L, Phenotypic and genetic diversity of *Paenibacillus azotofixans* strains isolated from the rhizoplane or rhizosphere soil of different grasses. J Appl Microbiol, 1998; 84: 216-226.
34. Vargas-Garcia M, Lopez M, Elorrieta M, Suarez F & Moreno J, Influence of nutritional and environmental factors on polysaccharide production by *Azotobacter vinelandii* cultured on 4-hydroxybenzoic acid. J Ind Microbiol Biotechnol, 2001; 27: 5-10.
35. Clementi F, Crudele MA, Parente E, Mancini M & Moresi M, Production and characterisation of alginate from *Azotobacter vinelandii*. J Sci Food Agric, 1999; 79: 602-610.
36. Asami K, Aritomi T, Tan Y & Ohtaguchi K, Biosynthesis of polysaccharide alginate by *Azotobacter vinelandii* in a bubble column. J Chem Eng Japan, 2004; 37: 1050-1055.
37. Mathlouthi M & Koenig JL, Vibrational spectra of carbohydrates. In: *Anonymous Advances in carbohydrate chemistry and biochemistry*. (Elsevier) (1987) 7.
38. Papageorgiou SK, Kouvelos EP, Favvas EP, Sapalidis AA, Romanos GE & Katsaros FK, Metal–carboxylate interactions in metal–alginate complexes studied with FTIR spectroscopy. Carbohydr Res, 2010; 345: 469-473.
39. Okabe E, Nakajima M, Murooka H & Nishizawa K, Investigation of carbon and phosphorus sources in cultural media of a selected strain of alginate-producing *Azotobacter vinelandii*. Journal of fermentation technology, 1981; 59: 1-7.
40. Page WJ &Sadoff HL, Relationship between calcium and uroinic acids in the encystment of *Azotobacter vinelandii.* J Bacteriol, 1975; 122: 145-151.
41. Nunez C, Moreno S, Soberon-Chavez G & Espin G, The *Azotobacter vinelandii* response regulator AlgR is essential for cyst formation. J Bacteriol, 1999; 181: 141-148.