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Research Article

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Metabolic potential of *Azotobacter* alginate producers and sustainable alternatives for alginate extraction

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Abstract

This research aimed to assess the metabolic activities of *Azotobacter vinelandii* DT1 strain by assaying its alginate synthesizing enzymes and modifying genes, synthesize alginate from the agricultural residues, and eradicate the pathogenicity and risk associated with *Pseudomonas aeruginosa* alginate. Preliminary screening for alginate production revealed the presence of different alginate products. High-performance liquid chromatography (HPLC) confirmed the existence of varying alginate concentrations, such as sodium alginate (2.42754×10^{-1}g/ 100 ml), calcium alginate (1.09597×10^{-1}g/ 100 ml), acid alginate (1.39420×10^{-2}g/ 100 ml), alginate oligosaccharide (8.20576×10^{-2}g/100 ml), and potassium alginate (9.78836×10^{-2}g/ 100 ml). These were accompanied with the corresponding alginate synthesizing enzymes; mainly GDP-Mannose dehydrogenase (23.77± 0.13 U/ ml); glycosyltransferase (9.68± 0.53 U/ ml), phosphomannomutase (266.09± 0.16 U/ ml), mannose phosphate isomerase (95.87± 0.51 U/ ml), alginate lyase (24.50± 0.95 U/ ml), and mannuronan epimerase (49.93± 0.82 U/ ml). In this study, the expression of alginate-modifying genes such as alginate lyase and GDP-Mannose dehydrogenase amplicons of *Azotobacter vinelandii* DT1 strain corresponding to 766 bp, 43 ng and 600 bp, 33 ng molecular weights of the fast DNA marker; justified the synthesis of different alginate products. *Azotobacter* alginate synthesis using a low-cost substrate (*i.e.*, corn cobs) and completely non-pathogenic bacteria (*Azotobacter vinelandii* DT1) may be desirable compared to the pathogenicity risks and poor jellying qualities linked to *Pseudomonas* alginate biosynthesis, its expensive production costs, and the adverse environmental effects of seaweed harvesting and processing.

Keywords: Alginate lyase, GDP-Mannose dehydrogenase, Mannose phosphate isomerase

1. Introduction

Understanding the metabolic activity involved in bacterial alginate biosynthesis is vital to maximizing alginate polysaccharide production in enormous-scale biological reactors. Alginates are polysaccharides comprising varying amounts of β, D-mannuronate and its C-5 epimer -L-glucuronate joined by 1,4 glycosidic linkages. Alginate, which comprises 40 % of seaweed's dry weight, is anticipated to have a structural role similar to cellulose in land-dwelling florae (Núñez *et al*., 2022). Alginate applications include wound dressings in the medical; pharmaceutical, and biotechnology sectors (Gheorghita *et al*., 2020), controlled release of medicinal products, enzymatic agents or cells, and scaffolds for tissue creation, because of its desired material characteristics and putative biological compatibility (Farshidfar *et al*., 2023).

There are several applications of alginate due to its unique physical qualities. Abbas *et al*., (2024) reported the hemocompatibility and antibacterial potentials of nano-gold-cored alginate against the anaerobic bacteria. Alginate cross-linked hydrogel, its medicinal properties with amoxycillin drug and water discharge property of the medication during wound healing was also analysed by Kannan *et al*., (2024). However, considerable advancements in these applications are not possible due to expensive production costs and adverse environmental effects of seaweed harvesting and processing. Strains of *P. aeruginosa* and *Azotobacter vinelandii* release extracellular polyuronides that closely mimic alginic acid discovered from brown algae (Vassoler, 2022), which opened up an intriguing potential economic route for a microbial polysaccharide (Remya *et al*., 2022). Employing seaweeds as alginate manufacturing sources is challenging due to the difficulty in attaining the specific physicochemical qualities needed to produce alginates beneficial for specialized applications, thus only a few seaweed species is believed to be suitable for extraction, which results in low alginate production (Urtuvia *et al*., 2017). Furthermore, due to alginate's connection to the pathogenicity of *P. aeruginosa* strains, microbial alginate has attracted lots of interim medical significance (Varaprasad *et al*., 2016). Thus, a microbial-optimized production process for this beneficial molecule is required due to the rapid development of this polymer's medicinal applications and the discovery of its special immunological features (Gheorghita *et al*., 2020). The objectives of this research were to assess the metabolic activities of *Azotobacter vinelandii* DT1 strain by assaying its alginate synthesizing enzymes and modifying genes, synthesize alginate from the agricultural residues, and eradicate the pathogenicity and risk associated with *P. aeruginosa* alginate.

2. Materials and methods

2.1. Isolation of *Azotobacter* species

Five samples of garden soil (2 g each) from a depth of 10-15 cm were collected from different locations in the biological garden of Fountain University's Osogbo, Nigeria, treated with crushed grape extract and 1 % sodium benzoate to support the growth of the *Azotobacter* species. The crushed grape extract was obtained from previously washed grape fruits, processed using a blender, and filtered to remove the pulp and seeds to release the extract. The treated soil sample (1 g) was transferred into sterile dist. water (10 ml); and subjected to serial dilution. Approximately, 0.1 ml of 10^{-2} diluent was spread aseptically on the surface of azotobacter agar medium (HIMEDIA) using spread plate method and incubated at 37 $^{\circ}$C for 48 h (Shaikh and Shakir, 2018). *Azotobacter* colonies of about 4-8 mm diameter with distinctive morphology, including colony shape, size, color, and consistency (raised, convex, smooth, regular and irregular, white, creamy, mucoid and viscous, transparent, translucent, opaque, glistening or dull) were chosen and purified by subsequent streaking on Luria-Bertani agar (LB)

medium; which was composed of (in g\ 1 dist. H_2O): yeast extract: 5; tryptone: 10; NaCI: 5; glucose: 2, and bacteriological agar: 10. The purified *Azotobacter* species were stored on LB agar slants and refrigerated at 4 °C for subsequent analysis.

2.2. Morphological and biochemical characterization of *Azotobacter* species

The *Azotobacter* species were identified according to the methodology conducted by Shaikh and Shakir, (2018). Colonial morphologies such as colony size, shape, surface appearance (*i.e*., elevation, margin, and transparency), and pigment formation were observed on Azotobacter agar and LB agar after 24 h incubation. Pure cultures of *Azotobacter* species were Gram stained and observed microscopically under an oil immersion objective (x100). The vegetative morphology, including cell form, organization, and Gram reaction, was documented. The pure cultures of *Azotobacter* species were subjected to biochemical analyses using several assays, including motility, oxidase and catalase production, nitrate reduction, citrate utilization, indole, and starch hydrolysis, according to Boone and Castenholz, (2001).

2.3. Metabolic activities of *Azotobacter* species

2.3.1. Screening of *Azotobacter* species for alginate-producing potential

Pure cultures of *Azotobacter* species (8 isolates) were screened for alginate production using LB agar. A loopful of 24 h-old *Azotobacter* culture cells was inoculated into LB-agar plates, and incubated at 37 °C for 24 h. The cultures producing heavily mucoid colonies with distinct pigmentation were selected as alginate producers.

2.3.2. Assay for *Azotobacter* species alginate-degrading potential

Azotobacter species were screened for alginate degrading potential, which determined their ability to utilize alginate as a major carbon source for their metabolic activity. About 5 µl of 24 h old Luria-Bertani culture broth [(tryptone (0.25g/ 100 ml), yeast extract (0.125g/ 100 ml), NaCl (0.125g/ 100 ml), and corn bran (0.025g/ 100 ml)] of each selected *Azotobacter* species was streaked at the center of Luria-Bertani agar plate and incubated at 30 °C for 48 h. After incubation, the cultured media were flooded with Lugol's iodine for 3 min. to determine the appearance of distinct clearance zone, thus, indicating alginate lyase's enzymatic activity (Sawant *et al*., 2015). Thus, one unit of enzyme activity was defined as the quantity of a biological catalyst required to enhance the clearance zone by 0.1 mm^2/ min. (Wang *et al*., 2017).

Furthermore, the alginate lyase metabolic activity was established using an ultraviolet absorption technique according to the modified method reported by Tavafi *et al*., (2017). After incubating the cultured Luria-Bertani broth for 24 h, 300 µl of the supernatant obtained through centrifugation at 8000 rpm for 10 min. were withdrawn to determine the initial alginate lyase activity spectrophotometrically at 235 nm. Ammonium sulfate (80 %) was used to precipitate 200 ml of the supernatant, and kept at 4 °C. The unprocessed enzyme obtained after 18 h through spinning for 15 min. at 15,280 rpm was liquefied in 1 ml phosphate buffered saline, and the resulting solution (0.3 ml) was combined with 2.7 ml of Tris-HCl (50 mM, pH 7.0) containing sodium alginate (0.5 %) as a substrate. After incubation at 30 °C for 6 min., the mixture was heated in boiling water for 4 min. to terminate the reaction process. One unit of enzyme activity was defined as the quantity of a biological catalyst enhancing the absorbance at 235 nm by 0.1/ min.

2.4. Microbial synthesis of alginate

Alginate-promoting medium (Luria-Bertani broth; LB) composed of tryptone (0.25g/ 100 ml), yeast extract (0.125g/ 100 ml), NaCl (0.125g/ 100 ml), and glucose (0.025g/ 100 ml) was utilized for alginate production assay (Sezonov *et al*., 2007). However, the carbon source (glucose) was substituted individually with the agricultural residue (*i.e*., corn bran or rice

bran), and the medium's pH was adjusted to 7 before sterilization. The alginate-producing *Azotobacter* spp. with an inoculum size of 1.1×10^2 cfu/ ml were inoculated individually into separate sterile broth media (Luria-Bertani-corn bran and Luria-Bertani-rice bran) and incubated at 37 °C for 24 h. After incubation, each cultured medium was centrifuged at 1,500 rpm for 10 min.; and the resulting supernatant was subjected to a preliminary analysis of alginate using the Bitter-Muir method (Kavitha *et al*., 2019) and thin layer chromatography (TLC). Furthermore, the effect of different extraction techniques on alginate yield was also analyzed.

2.4.1. Preliminary analysis of alginate

The Bitter-Muir method was utilized for preliminary analysis of alginate with slight modifications (Kavitha *et al*., 2019). The supernatant (0.5 ml) was gradually incorporated into 2 ml of 0.95 % $Na_2B_4O_7.10H_2O$ in 98 % H_2SO_4 in an ice bath at 80 °C, the resulting solution was heated at 100 °C for 10 min. and chilled in a frost bath for 10 min. Furthermore, 0.1 ml of 0.01 % pyridine in ethanol was incorporated into the solution, heated at 100 °C for 15 min., subsequently cooled in a frost bath at 25 °C, and the optical density at 530 nm was determined using Microfield UV-Spectrophotometer (MF-752N, England) . The amount of alginate was determined using the alginate oligosaccharide standard curve. Furthermore, the supernatants (3 ml each) were spotted individually on a silica gel TLC plate (Merck, Germany) and purity of the synthesized alginate was analyzed compared to the standard. The TLC plate was exposed to a running solvent system composed of acetic acid-water-butanol (6:1:4 v: v: v), according to the methodology conducted by Sawant *et al*., (2015). After spraying with 10 % (v/v) tetraoxosulphate (IV) acid in ethanol, the developed spots were detected by heating the TLC plate at 90 °C for 15 min. The alginate oligosaccharide solution (1.25 mg/ ml) was used as a standard. The production medium recording the highest alginate concentration was further subjected to confirmatory analysis of alginate using high-performance liquid chromatography (HPLC) and analytical analysis to ascertain the activities of the alginate-modifying enzymes.

2.4.2. Effect of alginate extraction techniques

The effect of extraction solvents on alginate yield was determined using different extraction methods. Before extraction, the supernatant was de-pigmented with 85 % ethanol until it become colorless. The acid alginate, sodium alginate, and calcium alginate extracts were prepared based on the methodology reported by Yudiati and Isnansetyo, (2017).

i. Acid alginate extraction

Acid alginate was extracted with 2 % Na_2CO_3 using water bath extraction technique at 70 °C for 2 h. The mixture was filtered, precipitated using HCL at pH < 1, and centrifuged at 3.200 rpm for 20 min. The supernatant was discarded, while the pellet was rinsed with 100 % ethanol (1:1) and then filtered.

ii.Sodium alginate extraction

Sodium alginate was extracted using an overnight magnetic stirring technique with 5 % Na_2CO_3 and 50 mM EDTA. The solution was filtered after 24 h, and the pellet obtained was added to potassium chloride (0.13 M) mixed with 96 % ethanol in 1:1 volume. The solution was stirred vigorously and centrifuged for 5 min. at 3500 rpm.

iii. Calcium alginate extraction

Extraction of calcium alginate was conducted by adding 0.2 N HCl to the supernatant and immersion in a water bath (60 °C) for 2 h. The pellet was filtered at pH 7 but regulated to pH 2 by rinsing with absolute ethanol and spun at 3000 rpm for 15 min. Finally, the obtained alginate was oven-dried at 60 °C for 24 h. The alginate yield (%) was determined by comparing the dried weight before and after extraction.

2.4.3. Confirmatory analysis of alginate

The alginate assay was performed to confirm the characteristics of the different alginate products

synthesized using HPLC according to the modified methodologies of Awad and Aboul-Enein, (2013). The supernatant obtained from the best alginate production medium was filtered using 0.22 µm filter paper, and the standard alginates (*i.e*., potassium alginate, sodium alginate, calcium alginate, alginic acid, and alginate oligosaccharide) of varying concentrations were transferred into the HPLC system for standardization and correlation coefficient establishment. The extracts (20 µl) were injected into the HPLC system (AGILENT 1200 SERIES) following similar process as the standard mixtures. Chromatographic separation utilized an isocratic elution method with a mobile phase of buffer solution consisting of 0.5 ml phosphoric acid in 1:1 dist. water adjusted to pH 7 with sodium hydroxide solution. The flow rate was 0.70 ml/ min. to ensure complete separation, and the extracted alginate was detected with an AGILENT 1260 detector at 200 nm.

2.5. Analytical analysis of alginate-modifying enzymes

The supernatant obtained from the best alginate production medium was spun down for 5 min. at 13,000 rpm to separate the liquid and solid fractions. The liquid fraction was collected in the pre-cleaned borosilicate beaker and further analyzed for the presence of GDP-mannose dehydrogenase, glycosyltransferase, phosphomannomutase, and mannose phosphate isomerase enzymes. In contrast, the un-centrifuged production broth was analyzed for mannuronan epimerase activity required for the production of alginate precursors and products.

i. GDP-mannose dehydrogenase assay

GDP-mannose dehydrogenase metabolic activity was evaluated by monitoring the increased absorbance (A340) using Microfield UV-Spectrophotometer (MF-752N, England) according to the modified methodology of Zhang *et al*., (2016). The enzymatic assay mixture (200 µl) contained 0.33 mM GDP-Mannose, 1 mM NAD+, 100 mM Tris-HCl buffer (pH 8.3), and purified supernatant (40 µg). The reaction was carried out in triplicate for 20 min., and kinetic parameters (Km) of *Azotobacter vinlandii* GDP-mannose dehydrogenase (AvGMD) and catalytic rate were measured under optimal conditions.

ii. Glycosyltransferase assay

The glycosyltransferase activity required for alginate polymerization was quantified in the supernatant using a glycosyltransferase activity kit (R&D Systems, Inc., a Bio-Techne Brand; Catalog # EA001) through a colorimetric technique according to the modified methodology of Rodríguez-Carrio *et al*., (2020). The glycosyltransferases activity was evaluated using 4 mM 4-Nitrophenyl-N-acetyl-b-d-glucosaminide (acceptor substrate) and 10 mM uridine 5′-diphospho- galactose (donor substrate) at 37 $^{\circ}$C for 30 min. Furthermore, nucleotidase ENTPD3/CD39L3 was used as a coupling phosphatase to eliminate the inorganic phosphate quantitatively from the nucleotide diphosphate (UDP) produced during glycosyltransferase reactions. The released inorganic phosphate was detected based on a colorimetric signal at 620 nm by the malachite green phosphate detecting reagents. Concentration of inorganic phosphate generated by the phosphatase was equivalent to the nucleotide sugar utilized during the reaction, which reflected the kinetics of the glycosyltransferase reaction that was extrapolated from an inorganic phosphate standard curve with known concentrations. Each sample was analyzed in triplicate, and the phosphate concentration equivalent generated per minute (M P/ min.) expressed the glycosyltransferase activity.

iii. Phosphomannomutase assay

Phosphomannomutase activity was analyzed through a spectrophotometric technique at 340 nm using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer) at 32 $^{\circ}$C to assay for the reduction of $NADP^{+}$ to NADPH. The reaction mixture (0.5 ml) comprised of 20 mmol/ l HEPES buffer (pH 7.5), NaCl (150 mmol/ l), $MgCl_2$ (5 mmol/ l), NADP+ (0.25 mmol/ l) and Man-1-P (0.6 mmol/ l), phosphoglucose

isomerase (0.010 mg/ ml), yeast glucose 6-phosphate dehydrogenase (0.01 mg/ ml), phosphomannose isomerase (0.0035 mg/ ml), Glc-1,6-P2 (0.03 mmol/ l) (instead of Man-1,6-P2) according to the procedure of Andreotti *et al*., (2013).

iv. Mannose phosphate isomerase assay

Mannose phosphate isomerase activity was measured by evaluating the amount of fructose -6-phosphate generated per unit time. The reaction mixture containing 0.5 mM $CoCl_2$ (15 µl), supernatant (25 µl), 50 µl of 15 mM Mannose-6-Phosphate, 20 mM phosphate buffer (10 µl, pH 7.5) was stored at 70 °C for 5 min. and further cooled on ice for 10 min. to slow the reaction (Sigdel *et al*., 2015). The presence of fructose-6-phosphate in the reaction mixture was analyzed using cysteine carbazole-sulfuric acid technique according to the modified methodology of Dische and Borenfreund, (1951) by adding 1.5 % cysteine hydrochloride solution (2 µl), a mixture of water (19 µl) and concentrated sulfuric acid (45 µl) to 10 µl of the cooled reaction mixture. Immediately, 0.12 % alcoholic solution of carbazole (2 µl) was incorporated into the mixture, shaken, and kept at a stationary condition at 28 °C. The appearance of purple color after 10 min. indicated the presence of fructose-6-phosphate. The product (fructose-6-phosphate) yield was quantified using a spectrophotometer (560 nm), where one unit of mannose phosphate isomerase activity was defined as the quantity of fructose-6-phosphate produced per minute.

v. Mannuronan epimerase assay

Mannose epimerase activity responsible for increasing alginates' α-l-guluronic acid (G) content in vitro was analyzed according to the modified methodology of Gevaert *et al*., (2019). *Azotobacter vinelandii* DT1 cultured broth (100 ml) obtained from the production medium was cooled to 20 °C, isopropyl -D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and stored overnight on a rotary shaker (200 rpm) at 20 °C to promote production of the enzymes. Centrifugation of the reaction mixture was carried out at 4 °C for 25 min. and 10,000 rpm to separate the cells, and the resulting pellet was chilled and kept for 60 min. at -20 °C. The pellet was reconstituted in an extraction buffer (8 ml), which contained imidazole (10 mM), 500 mM sodium chloride, 1 mg/ ml of lysozyme, and phenylmethane sulfonyl fluoride (100 µM) in a 50 mM sodium phosphate buffer (Boston BioProducts) with a pH of 7.4, and the suspension was chilled on ice for 30 min. for enzyme extraction and purification. Furthermore, the cells were subjected to sonication using Branson SFX150 digital sonifier cell disruptor, and the cell debris was removed by spinning for 15 min. at 10,000 rpm and 4 °C. The soluble protein supernatant was filtered using a 0.2 m polyethersulfone membrane filter, and the protein concentration was evaluated at 280 nm using the Thermo Scientific NanoDrop 2000c Spectrophotometer (Marshall Scientific, USA).

2.6. Molecular characterization of *Azotobacter* GDP-mannuronic dehydrogenase (*Alg D*) and Alginate lyase (*Alg L*) genes

i. DNA extraction and quantification

Genomic DNA of *Azotobacter vinelandii* DT1 and *Azotobacter chromococcum* CC12 was extracted using the Quick-DNA Fungal/ Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The bacterial cells (50-100 mg wet weight) were transferred into a ZR BashingBead™ lysis tube (0.1 and 0.5 mm) containing Bashing bead buffer (750 µl). The tube was kept in a bead beater (disruptor genie) and processed for 20 min.; thus, the ZR BashingBead™ lysis tubes (0.1 and 0.5 mm) were centrifuged at 10000 rpm for 1 min. The supernatant (400 µl) was poured into a collecting tube with a Zymo-SpinTM III-F Filter and spun at 8000 rpm for 1 min., the Zymo-Spin™ III-F filter was discarded, and the genomic lysis buffer (1200 µl) was carefully mixed with the obtained filtrate. Furthermore, the mixture (800 µl) was transferred into a Zymo-Spin IICR column in a collection tube, spun at 10,000 rpm for 1 min., and the flow-through was discarded. The Zymo-Spin™ IICR column was transferred into a new collection tube containing DNA

pre-wash buffer (200 µl), spun at 10,000 rpm for 1 min., and the flow-through was discarded. Then, 500 µl of g-DNA wash buffer was further added to the column in a collection tube and centrifuged at 10,000 rpm for 1 min. The column was dispensed into a clean microcentrifuge tube (2 ml), containing DNA elution buffer (50 µl). The mixture was further centrifuged at 10,000 rpm for 30 sec. to elute the DNA, and the quantity and quality of the extracted DNA were determined using a nanodrop UV-Vis Spectrophotometer (Thermo Scientific). The system was blanked using DNA elution buffer (1 µl), 1 µl DNA was placed on the pedestal, and the concentration (ng/ µl) was recorded using the A260/ 280 ratio and A260/ 230 ratio of the sample (Jimoh *et al*., 2013).

ii. Polymerase chain reaction (PCR) analysis of *Alg D* and *Alg L* genes

Azotobacter vinelandii DT1 and *Azotobacter chromococcum* CC12 isolates were subjected to PCR analysis for GDP-Mannuronic dehydrogenase (*Alg D*) and Alginate lyase (*Alg L*) genes using 2X Master Mix (NEB, Catalogue No. M0486) and forward and reverse primers of *Alg D* and *Alg L* genes (Inqaba, South Africa) according to the manufacturer's instruction. PCR water (nuclease-free, 4.75 µl) was added to 1 µl of genomic DNA template, 0.25 µl each of forward and reverse primers, and 6.25 µl of PCR Master Mix in a sterile microcentrifuge tube. The solution was mixed gently, 50 µl of mineral oil was added to avoid evaporation, and the mixture was exposed to thermal cycling conditions using the Eppendorf Mastercycler (Nexus gradient 230). The PCR thermal cycling conditions for *Alg D* gene included initial denaturation of double-stranded DNA by heating at 94 °C for 5 min.; annealing of primers to the template at 57 °C for 1 min., and extension at 68 °C for 1.5 min. After the first cycling, cyclic denaturation at 94 °C for 30 sec. was repeated till the final extension of new DNA at 68 °C for 10 min. for 35 cycles. The PCR thermal cycling conditions for *Alg L* gene included initial denaturation of double-stranded DNA by heating at 94 °C for 5 min.; annealing of primers to the template at 55 °C for 1 min., and extension at 68 °C for 1.5 min. After the first cycling, cyclic denaturation at 94 °C for 30 sec. was repeated till the final extension of new DNA at 68 °C for 10 min. for 35 cycles. The primer sequences of *Alg D* and *Alg L* forward and reverse primers (Inqaba, South Africa) used for PCR analysis are as follows;

Alg D (forward primer): 5-ACGAAGTGGTGGCGAGTTC -3

Alg D (reverse primer): 5-TGGTGTGCGGCATGAAGC -3

Alg L (forward primer): 5-ATGAAAACGTCCCACCTGATCCG -3

Alg L (reverse primer): 5-TCAACTTCCCCCTTCGCGGC -3

After the amplification was conducted, 2 µl of each PCR products was resolved on agarose gel (l %) containing 5 µl of SafeView red stain. The amplicon (2 µl) was mixed with a loading buffer (1 µl); loaded onto the agarose gel and the power supply was adjusted to 100 volts for 25 min. For each complete cycle, a 10 kb molecular weight of Fast DNA ladder was used to determine the size of each PCR product with a control. The DNA bands were visualized with a short-wave ultraviolet trans-illuminator and photographed using a gel documentation system (E-BOX, Vilber Lourmat, Italy) (Jimoh *et al*., 2013).

2.7. Statistical analysis

The findings of this study were analyzed with GraphPad Prism 8.0.1 using one-way analysis of variance (ANOVA) with pairwise comparisons using Tukey's technique for the alginate metabolic enzymes activities and alginate yield to identify the significant difference among means of the triplicate analysis.

3. Results

3.1. Morphological and biochemical characteristics of *Azotobacter* species

Azotobacter species isolated from garden soil samples were characterized based on their colonial morphology on Azotobacter agar and LB agar and their corresponding biochemical characteristics. Colonial characteristics of *Azotobacter* species (8 isolates) such as shape and size (spherical, domed, raised, flat on the top, irregular and regular), appearance (off-white, white, creamy, viscous, and mucoid); consistency (transparent, translucent, and opaque colonies) are as shown on Table (1). Based on Gram reaction, the isolates were Gram-negative rods and identified biochemically as *Azotobacter nigricans*, *Azotobacter agilis*, *Azotobacter salinestris*, *Azotobacter chromococcum*, *Azotobacter armeniacus*, *Azotobacter tropicalis*, *Azotobacter beijerinckii*, and *Azotobacter vinelandii* using several assays including hydrolysis of starch, utilization of citrate and different carbon sources, oxidase and catalase activities, motility, nitrogen reduction, and indole production potential (Table 2).

3.2. Alginate-producing potential of *Azotobacter* species

The colonial morphology of *Azotobacter* species on LB agar also varied from greenish, orange, whitish, and off-white, with varying diameters between 1.0 and 1.2 mm (Table 3). Among the eight *Azotobacter* species screened for alginate-producing activities using LB agar, only two species (*Azotobacter vinelandii* DT1 and *Azotobacter chromococcum* CC12) with pigment formation expressed alginate-producing potentials (Table 3). Furthermore, colonial morphology of the strains on Luria-Bertani-corn bran and Luria-Bertani-rice bran medium was similar, but the colony sizes differed. *Azotobacter* species colony sizes on Luria-Bertani-corn bran agar were 1.2 mm, but 1.0 mm on Luria-Bertani-rice agar (Table 3). Thus, Luria-Bertani-corn bran medium, *Azotobacter vinelandii* DT1 and *Azotobacter chromococcum* CC12 strains with the most expansive colony sizes were utilized for alginate production.

3.3. Qualitative and quantitative analysis of extracellular alginate lyase

Two *Azotobacter* species, namely *Azotobacter vinelandii* DT1 and *Azotobacter chromococcum* CC12, which grew favorably on LB agar were analyzed for alginate-lyase-activity using iodine solution. *Azotobacter* cultured medium with distinct and prominent zones of clearance around the colonies, indicated the presence of alginate lyase while blue-black coloration in the non-hydrolyzed portion of the medium indicated alginate production. *Azotobacter vinelandii* DT1 expressed the most expansive zone of bluish-black coloration with a minimal zone of clearance, indicating minimal production of alginate lyase required for alginate degradation (Table 4).

3.4. Alginate quantification and characterization

There was a significant difference between the alginate concentration produced by *Azotobacter vinelandii* DT1 using LB broth (2 %) compared to the rice bran (1 %) and corn bran (2.85 %) media at $p< 0.05$ (Table 5). The effect of different extraction methods on alginate yield revealed the presence of sodium alginate, acid alginate, and calcium alginate (Table 6). TLC analysis revealed the ability of *Azotobacter vinelandii* DT1 to produce pure alginate products in a more significant proportion attributed to the fact that the *Azotobacter vinelandii* DT1 alginate retention factor (R_f) was 0.79, which was relatively close to the 0.8 R_f of the alginate oligosaccharide used as a standard, while *Azotobacter chromococcum* CC12 alginate R_f (0.62) differed significantly.

Confirmatory analysis using HPLC established the presence of five alginate products, including calcium alginate, sodium alginate, potassium alginate, alginic acid, and alginate oligosaccharide (Table 7).

3.5. Alginate synthesizing enzymes

Azotobacter vinelandii DT1 metabolic activity during the alginate synthesis was revealed by its ability to express genes required for the synthesis of alginate synthesizing enzymes such as GDP-mannose dehydrogenase, glycosyltransferase, phosphomannomutase, mannose phosphate isomerase, and mannuronan epimerase (Table 8).

Table 1: Colonial Characteristics of *Azotobacter* species

Isolate code	Shape	Appearance	Color	Elevation	Edge	Diameter (mm)
PT 1	Round	Mucoid	Cream	Raised	Entire	1.2
PT 2	Round	Opaque	White	Raised	Entire	1.1
PW 1	Round	Mucoid	Cream	Raised	Smooth	1.1
PW 2	Irregular	Non-mucoid	White	Flat	Undulated	1.1
CC 11	Round	Opaque	Off-white	Flat	Smooth	1.1
CC 12	Round	Opaque	Off-white	Flat	Smooth	1.1
CC 2	Irregular	Mucoid	Cream	Raised	Rough	1.2
DT 1	Round	Mucoid	Cream	Raised	Rough	1.2

Table 2: Biochemical characteristics of *Azotobacter* species

Biochemical reactions	PT1	PT2	PW1	PW2	CC11	CC12	CC2	DT1 +
Citrate	+	+	+	+	+	+	+	
Indole	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+
Starch hydrolysis	+	_	+	+	+	+	_	+
Catalase	+	+	+	+	+	+	+	+
Motility	+	+	_	+	+	+	_	+
Carbon sources utilized								
Sucrose	+	_	+	+	_	+	+	+
Fructose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Mannitol	_	_	_	+	_	+	_	_
Maltose	_	+	_	_	_	+	+	+
Species identity	*A. agilis*	*A. armeniacus*	*A. nigricans*	*A. tropicalis*	*A. salinestris*	*A. chromococcum*	*A. beijerinckii*	*A. vinelandii*

Where; + = Catalase and oxidase enzymes present, nitrate reduced, motile, citrate utilized, indole present (ability to convert tryptophan to indole), carbon utilized, starch hydrolyzed; - = Catalase and oxidase absent, nitrate reduction absent, non-motile, citrate unutilized, indole absent, starch un-hydrolyzed, carbon un-utilized

Table 3: Alginate-producing potential of *Azotobacter* species on Luria-Bertani, Corn bran, and Rice bran media

Isolates	Colonial morphology and diameter of alginate production				
	Shape/ Elevation/ Edge	Appearance/ Color	Zone of alginate production (mm) on LB agar	Zone of alginate production (mm) on LB -Corn bran agar	Zone of alginate production (mm) on LB-Rice bran agar
A. agilis PT 1	Irregular/ Flat/ Rough	Mucoid/ Off-white	1.2	1.2	1.0
A. armeniacus PT2	Irregular/ Flat/ Rough	Mucoid/ White	1.1	1.0	1.0
A. nigricans PW 1	Irregular/ Flat/ Rough	Mucoid/ Cream	1.1	1.1	1.0
A. tropicalis PW 2	Round/ Elevated/ Rough	Mucoid/ Cream	1.1	1.0	1.0
A. salinestris CC 11	Irregular/ Raised/ Rough	Mucoid/ Cream	1.2	1.1	1.0
A. chromococcum CC 12	Round/ Raised/ Smooth	Mucoid/ Orange	1.2	1.2	1.1
A. beijerinckii CC 2	Round/ Flat/ Smooth	Mucoid/ Cream	1.2	1.1	1.0
A. vinelandii DT 1	Round / Raised/ Smooth	Mucoid/ Green	1.2	1.2	1.1

Where; LB agar: Luria-Bertani agar medium

Table 4: Alginate lyase activity of *Azotobacter* species

Azotobacter species	Zone diameter of clearance (mm)[a]	Size of the bacterial colony (mm)[a]	Alginate lyase activity area (mm)[b]
A. vinelandii DT1	8.33± 0.48	10.00± 1.00	54.12
A. chromococcum CC12	19.27± 1.23	10.00± 1.00	291.50

Where; [a]Data given in column represent mean ± standard deviation (±SD); [b]Alginate lyase activity area of each bacterial strain was calculated as πD2/4 where D is a zone of diameter (mm) for the total zone area due to enzyme activity

Table 5: Preliminary quantification of *Azotobacter* alginate yield

Azotobacter species	Alginate concentration (%)		
	Luria-Bertani-Glucose medium	Luria-Bertani-Rice bran medium	Luria-Bertani -Corn bran medium
A. vinelandii DT1	2.00 ± 0.2^{a}	1.00 ± 0.0^{b}	2.85 ± 0.15^{c}
A. chromococcum CC12	1.23 ± 0.1	1.30 ± 0.1	$2.35 \pm 0.2^{a,b,c}$

Where; Data followed by the same superscript letters are significantly different at $p < 0.05$. Data are presented as mean ± SEM (standard error of the mean); n=3. Statistical level of significance analyzed by one-way ANOVA followed by Tukey post hoc Pairwise Multiple Comparison tests: $^{a}p< 0.05$ compared to Luria-Bertani-Glucose medium, $^{b}p< 0.05$ compared to Luria-Bertani-Rice bran medium, $^{c}p< 0.05$ compared to Luria-Bertani -Corn bran medium

Table 6: *Azotobacter* alginate products based on the extraction techniques

Azotobacter species	Extraction technique/Alginate products (%)		
	Acid alginate	Sodium alginate	Calcium alginate
A. vinelandii DT1	13.75 ± 0.75^{a}	19.00 ± 0.11^{b}	16.50 ± 0.33^{c}
A. chromococcum CC12	8.12 ± 0.14	10.60 ± 0.47	$12.45\pm 0.24^{a, b, c}$

Where; Data followed by the same superscript letters are significantly different at $p < 0.05$. Data are presented as mean ± SEM (standard error of the mean); n=3. Statistical level of significance analyzed by one-way ANOVA followed by Tukey post hoc Pairwise Multiple Comparison tests: $^{a}p< 0.05$ compared

Table 7: Confirmatory analysis of *Azotobacter vinelandii* DT1 alginate products

Retention time (min.)	Amount (g/100 ml)	Alginate products
16.546	2.42754×10^{-1}	Sodium alginate
17.725	1.39420×10^{-2}	Alginic acid
18.831	9.78836×10^{-2}	Potassium alginate
20.008	1.09597×10^{-2}	Calcium alginate
21.110	8.20576×10^{-2}	Alginate oligosaccharides
Total	3.61675×10^{-2}	-

Where; the recorded values were significantly different at $p< 0.05$. Data are mean ± SD (standard deviation), n= 3. The statistical level of significance analyzed by a one-way ANOVA is followed by Tukey post hoc pairwise multiple comparisons test

Table 8: Enzymatic activities during Alginate synthesis

Enzymes	Activity (U/ ml)
GDP-mannose dehydrogenase	23.7± 0.13
Glycosyltransferase	9.68± 0.53
Phosphomannomutase	266.09± 0.16
Mannose phosphate isomerase	95.87± 0.51
Mannuronan epimerase	49.93± 0.82

Where; the recorded values were significantly different at $p< 0.05$. Data are mean ± SD (standard deviation), n= 3. The statistical level of significance analyzed by a one-way ANOVA is followed by Tukey post hoc pairwise multiple comparisons test

3.6. Molecular characterization of *Azotobacter* alginate producers

The photometric measurement of *Azotobacter vinelandii* DT1 and *Azotobacter chromococcum* CCC12 nucleic acids was based on the intrinsic absorptive properties of the DNA and RNA, which absorb light with characteristics peaks at 1.94 nm and 5.23 nm, respectively (Table 9). Both alginate producers (*Azotobacter vinelandii* DT1 and *Azotobacter chromococcum* CC12) subjected to PCR analysis expressed PCR product specific for *Alg D* gene encoding GDP- mannose dehydrogenase, while only *Azotobacter vinelandii DT1* expressed *Alg L* gene encoding alginate lyase. Thus, *Alg D* amplicons had 33 ng molecular mass corresponding to the 600 bp of the Fast DNA marker, while the *Alg L* amplicon had 43ng molecular mass corresponding to 766 bp of the Fast DNA marker.

Table 9: Photometric measurement of *Azotobacter* species nucleic acids

Isolates	A260/A280	A260/A230	A260	A280	DNA concentration
A. vinelandii DT1	1.81	0.16	0.33	0.18	16.4
A. chromococcum CC12	1.85	0.28	0.20	0.11	10.2

4. Discussion

In this study, preliminary screening of *Azotobacter vinelandii* DT1 revealed the presence of alginate lyase, which plays an essential role in the degrading of alginate oligosaccharides (Zhu and Yin, 2015). *Azotobacter vinelandii* DT1 displayed the highest alginate-producing potential and minimal alginate degrading activity compared to *Azotobacter chromococcum* CC12 (Table 4). Availability of minimal alginate lyase activity is essential because this enzyme participates in clearing the periplasm for

alginate molecules originating from non-functional export complexes. Accordingly, free alginate molecules in the periplasm lead to amplified osmotic pressure that causes lysis, and lack of alginate lyase in alginate-producing microorganism affects its survival (Ertesvåg, 2015). *Azotobacter vinelandii* DT1 also expressed the potential to modify the alginate chain that failed to be secreted by the outer membrane porin (Shinabarger *et al*., 1991), due to the availability of alginate lyase. Alginate lyases are essential for enzymatic preparation of the functional oligosaccharides (Hay *et al*., 2013) that were identified in *Azotobacter vinelandii* and cleaved the glycosidic linkages of alginate by a β-elimination mechanism. This led to the formation of bacterial alginate products that contained unsaturated sugar units at the non-reducing end.

Preliminary screening of alginate synthesized using agricultural residues as substrates showed that corn bran is a rich source of starch with vitamins and minerals required for *Azotobacter vinelandii* DT1 growth, which eventually enhanced alginate production (Alleman *et al*., 2021). There was a significant difference between the alginate concentration produced by *Azotobacter vinelandii* DT1 using LB broth compared to the rice bran and corn bran media at $p< 0.05$ (Table 5). The effect of different extraction methods on alginate yield revealed the presence of sodium alginate, acid alginate, and calcium alginate. In contrast, sodium alginate had the highest yield compared to the other extraction techniques (Table 6), due to the addition of Ethylene diamine tetra-acetic acid, which acted as a chelating agent during sodium alginate extraction and, consequently, improved the extract yield.

Azotobacter vinelandii DT1 had the highest alginate production yield, which acted as a diffusion barrier for nutrients and oxygen (Cao *et al*., 2022). Confirmatory analysis using HPLC established the presence of five alginate products, including calcium alginate, sodium alginate, potassium alginate, alginic acid, and alginate oligosaccharide (Table 7). Varying concentrations of bacterial alginate (*i.e*., potassium alginate, sodium alginate, calcium alginate, alginic acid, and alginate oligosaccharide) obtained in this study confirmed the metabolic potential of *Azotobacter vinelandii* DT1 for the synthesis of alginates required for several commercial and industrial uses in the different sectors.

The industrial and environmental roles of *Azotobacter vinelandii* DT1, such as being biocompatible, biodegradable, economical, and ecofriendly in nature, were established by its ability to synthesize sodium alginate that is essential in removing the toxic heavy metal ions (*i.e*., wastewater treatment) (Saeed *et al*., 2016). *Azotobacter vinelandii* DT1 also revealed a medical potential by synthesizing calcium alginate required for cell immobilization support (Núñez *et al*., 2022), and potassium alginate mainly used in medicine and health care due to its functions in blood fats and sugars, and cholesterol-lowering (ALSamman and Sánchez, 2021). *Azotobacter vinelandii* DT1 alginate products with varying compositions and monomer structures obtained in this study may express their different properties and functions, which can be utilized for numerous purposes in industry (Xiao *et al*., 2023). Varying alginate configuration and concentration confirms the tendency of *Azotobacter vinelandii* DT1 to be an excellent alginate producer for the synthesis of hemocompatible, antibacterial, and effective nano-gold-cored alginate drug active against the anaerobic bacteria (Abbas *et al*., 2024), and alginate cross-linked hydrogel medication used during wound healing (Kannan *et al*., 2024).

Azotobacter vinelandii DT1 metabolic enzymes are responsible for the production of alginate intermediates that belong to the C2, C3, and C6 alginate groups, which serve as precursors for the synthesis of commercial alginates. Synthesis of mannose phosphate isomerase indicated the tendency of *Azotobacter vinelandii* DT1 to convert fructose-6-phosphate obtained from corn bran to mannose-6-phosphate (Zhu and Yin, 2015), while phosphomannomutase further converted mannose-6-phosphate to mannose-1-phosphate (Sigdel *et al*.,

2015). These are intermediates/ precursors for the synthesis of GDP-mannose that is catalyzed by the GDP-mannose pyrophorylase *via* the hydrolysis of GTP (Zielinski *et al*., 1991). *Azotobacter vinelandii* DT1 potential to synthesize alginate was attributed to the availability of GDP- mannose dehydrogenase enzyme required for transformation of the GDP-mannose obtained from the polysaccharide (agricultural residue) to GDP-mannuronic acid, which is the main alginate precursor (Table 8).

Furthermore, the expression of mannuronan epimerase (ME) (alginate-modifying enzyme) that facilitates the transformation of β-D-mannuronate to its epimer α-L-guluronate in alginate, confirms the metabolic potential of *Azotobacter vinelandii* DT1 to synthesize biotechnological devices for modifying and processing alginate of varying characteristics, since the biological and physical properties of alginate are revealed by M/G ratios and distribution patterns (Zhu *et al*., 2016). This establishes the availability of diverse G-distribution patterns being expressed during the bacterial cell life cycle (Ertesvåg, 2015). Moreover, it authenticates the availability of L-guluronic acid residues in *Azotobacter vinelandii* DT1 polysaccharide alginate, which originates from a post-polymerization reaction catalyzed by the mannuronan epimerase enzyme (Ci *et al*., 2021).

Most alginate biosynthetic genes (*Alg*) are assembled in a chromosomal region and headed by the *Alg D* gene. The *Alg* genes are organized in three operons, but *Alg D* gene required for monomer generation occupies the first operon (Núñez *et al*., 2022). In this study, expression of the GDP-mannose dehydrogenase (*AlgD*) gene by *Azotobacter vinelandii* DT1 confirmed the availability of an alginate biosynthetic gene responsible for the synthesis of an enzyme that catalysed the final irreversible step. This resulted in the formation of GDP-mannuronic acid required for the alginate polymerization mechanism.

Conclusion

Understanding the *Azotobacter vinelandii* metabolic pathways creates a prospect for the synthetic biology and bioengineering methods toward alginate biosynthesis, which displays anticipated material properties suitable for several biomedical and pharmaceutical applications. Although commercial alginate is obtained from different species of farmed brown seaweeds but due to variations in chemical configuration of alginate extracted from these diverse species; seaweeds are not considered a dependable source of alginic acid. Furthermore, many bacterial species such as *Pseudomonas* and *Azotobacter* are reported to produce alginate comparable in composition to the algal source. However, potential risks of pathogenicity and poor crystallizing properties associated with Pseudomonas alginate make *Azotobacter vinelandii* DT1 more suitable for alginate production.

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Conflict of interests

The authors declare no conflicts of interest.

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Author's Contributions

Conceptualization, S.J. and F.A.; Roles/Writing - original draft, S.J.; Data curation, S.J.F.A. and K.A.; Formal analysis, S.J. and F.A.; Investigation, S.J. and F.A.; Methodology, S.J., F.A., K.A., R.B. and T.S.; Project administration, S.J., F.A., K.A. and R.B.; Software, S.J.; Resources, F.A.; Supervision, S.J. and K.A,; Visualization, S.J. and K.A.; Validation, S; J., F.A. and K.A.

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