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Condition Optimization for Phosphate Solubilization by *Kosakonia cowanii* MK834804, an Endophytic Bacterium Isolated from *Aegle marmelos*

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A B S T R A C T

In the present study endophytic bacteria were isolated from *Aegle marmelos* and were screened for phosphate solubilizing activity. The isolate AP01 showed the ability to solubilize inorganic tricalcium phosphate and thus was characterized. Phenotypic, biochemical and genotypic analysis revealed, the isolate to be *Kosakonia cowanii*, a gram –ve proteobacterium of family enterobacteriaceae with accession no. MK834804, of the three media viz. Pikovskaya's agar (PKVA), National Botanical Research Institute's phosphate growth medium (NBRIP) and NBRIP-BPB (Bromo Phenol Blue) agar media tested for Phosphate solubilizing efficiency, the isolate showed high Phosphate solubilizing Index (PSI 4.5) in PKVA medium. Whereas, under submerged fermentation the isolate showed highest phosphate solubilization in NBRIP medium (70.200µg/ml) on 4th day with maximum drop down of medium pH (3.6). Optimum solubilization of phosphorus was observed at temperature 37ºC (70.403µg/ml), pH 8 (71.748 µg/ml) with 0.5 % ammonium sulphate (79.76µg/ml) and 2% Lactose (87.283µg/ml) on 4th day of incubation period as optimal nitrogen and carbon sources respectively. Release of various organic acids i.e. oxalic acid, malic acid, tartaric acid and gluconic acid into the medium was characterized by High Performance Liquid Chromatography (HPLC), during phosphate solubilization by the isolate.

Introduction

Chemical fertilizers from the last few decades have been as a source of nutrients for crop plants. After nitrogen, the next important macronutrient required for plant growth is phosphorus (Bakhshandeh *et al.*, 2015). Recently, farmers are using large amounts of phosphatic fertilizer but only 10-25% is available to the plant rest become immobilized, leading to frequent input of these fertilizer making the process too cost effective as well as affect adversely to the soil environment (Bhattacharyya and Jha, 2012). Due to its low solubility by forming precipitates with some ions like iron, aluminum, calcium etc. it is unavailable to plants thereby limiting the plant growth (Liu *et al.*, 2014). So, for the better growth of the plant the phosphorus fixed in the soil must be solubilized and mobilized. There are many reports on soil microorganisms acting as bio-

phospho inoculants, but the use of endophytic microorganisms does have the better ability to solubilize this insoluble form of phosphorus than the soil or facultative microorganisms due to long co-evolutionary process within the host (Rosenblueth and Martínez-Romero, 2006). They make phosphorus (P) available to plants by secretion of low molecular weight organic acids thereby lowering the pH, which release the bound phosphate by blocking the P adsorption site in the soil particle (Rathi and Gaur, 2016; Pande *et al.,* 2017). The use of phospho-endobacteria alone or along with reduce amount of phosphate fertilizer could lead to organic and sustainable cultivation by reducing environmental pollution (Bakshandeh *et al.,* 2015). With this aim, the present study is concerned with isolation of endophytic bacteria from different parts of well-known medicinal plant i.e. *Aegle marmelos* (Rutaceae) and characterization for phosphate solubilizing capabilities *in vitro* in different phosphatic media by analysis of organic acids to a possible extent for biotechnological exploitations of untapped microorganisms.

Materials and Methods

Media and chemicals used

Nutrient agar, Pikovskaya's broth and agar media, National Botanical Research Institute's Phosphate Growth Medium (NBRIP), Bromo Phenol Blue (BPB), KB009B1, KB009C kit and Hi25 Enterobacteriaceae Identification kit (KB003) strips were procured from HiMedia Pvt. Ltd. Mumbai, India and prepared as per manufacturer's instructions and used in the study.

Sample collection

Infection free healthy twigs of *Aegle marmelos* (Bael) were collected from the garden [Odisha University of Agriculture and Technology, Bhubaneswar (India)], brought to the laboratory in sterile poly bags. Endophytic bacteria were isolated following a standard protocol (Panigrahi *et al.,* 2018). All the selected isolates were pure cultured and were maintained at 4˚C till further use.

Preliminary screening of endophytic bacteria for phosphate solubilization

Phosphate solubilizing activity of the isolates was qualitatively tested on Pikovskaya's agar, National Botanical Research Institute's Phosphate (NBRIP) and NBRIP-bromo phenol blue (NBRIP+0.025gm/l bromo phenol blue) agar medium by following the methods of Panigrahi *et al.,* (2018). Each of the medium containing tricalcium phosphate ($Ca_3(PO4)_2$) as the insoluble phosphate source. Formation of dissolution zone around the colonies is indicative of positive phosphatase activity and the Phosphate Solubilization Index (PSI) was determined by using the formula of (Premono *et al.,* 1996; Atekan *et al.,* 2014).

$$PSI = \frac{\text{Halo Zone + Colony Diameter}}{\text{Colony Diameter}}$$

Quantitative analysis of phosphate solubilizing efficiency of the isolate

Quantitative estimation of phosphate solubilization was carried out in 100 ml of Pikovskaya's Broth (PKVB) (pH 7) and NBRIP broth medium (pH 7) in 250 ml Erlenmeyer flask inoculated in triplicate with 1 ml of active culture of selected endophytic bacterial isolate AP01 which showed highest solubilization index in the solid medium. The flasks were then incubated at 37±2˚C for 11 days. An aliquot of 5ml was taken out from each flask at 24 h intervals, the pH was measured and then centrifuged at 6,000 rpm for 20 minutes and the clear supernatant was used for determination of available phosphorus concentration spectrophoto-metrically by the phospho-molybdenum method (Holman, 1943) at 660 nm.

Uninoculated medium served as control. The Potassium dihydrogen phosphate was used to make standard curve for determination of concentration of soluble phosphorus. The experiments were conducted in triplicates and values were expressed as their mean values and standard error of mean. Highest activity observed with the medium was considered as the suitable medium and was taken for further characterization and optimization of the isolate. The suitable medium was then supplemented with Aluminum phosphate ($AlPO_4$) & Ferric phosphate ($FePO_4$) to check the efficiency of the isolate to solubilize these inorganic phosphates.

Condition optimization for phosphatase activity of the isolate AP01

Both physical (incubation day, pH and temperature) and nutritional (varied carbon and nitrogen source) parameters were optimized for maximum solubilization of phosphorus in the NBRIP medium by the isolate in triplicates by following the procedure of Holman, (1943). Optimum temperature and pH for maximum phosphate solubilization was studied by culturing the selected isolate at different temperature (30, 37, 40, 45, 50º C) and pH (pH 4-12) and the assay was done at optimal day of incubation. Effect of various nitrogen sources (sodium nitrate, potassium nitrate, ammonium molybdate, peptone, urea, calcium nitrate, ammonium sulphate, asparagine) at varied concentration (0.1%, 0.3%, 0.5%, 1% w/v) and carbon sources (mannitol, dextrose, maltose, fructose, lactose, sucrose, trehalose, arabinose) at different concentration (0.5%, 1.0%, 1.5%, 2.0% w/v) on solubilization of phosphorus by the isolate AP01 was studied by supplementing to the NBRIP medium and assay was made by the method of Holman (1943).

Organic acid analysis

For the analysis of organic acid, the isolate AP01 was inoculated in 50ml of NBRIP medium and was incubated at 37º C upto 4th day. The cells were then harvested and centrifuged at 6000 rpm for 20 min. The supernatant was then filtered through 0.2 µm Syringe filter and was subjected for HPLC analysis. The identification of organic acids was performed using an Agilent 1260 Infinity series HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) ZORBAX 300Extend- C18 (250 mm×4.6 mm, 5 µm) column was used (Agilent Technologies, Inc.). The filtered supernatant (20 µl) was injected into the HPLC unit using a glass syringe with 8 mM H_2SO4 with a flow rate of 0.6 ml min^{-1} at 25ºC. Organic acid concentrations in the sample were determined with the help of RI detector. The unknown organic acids present in the supernatant were determined by comparing with the retention time of peaks of standard organic acids like tartaric acid, oxalic acid, malic acid, gluconic acid and citric acid.

Phenotypical and Biochemical characterization of AP01

Phenotypically the isolate AP01 was characterized on Nutrient Agar (NA), Pikovskaya's Agar (PA), MacConkey and NBRIP agar plates and were analyzed microscopically by Grams staining and Scanning Electron Microscopic image. The strain AP01 was characterized biochemically by using HiCarbo Kit (KB009B1 / KB009C) and Hi25 Enterobacteriaceae Identification kit (KB003) obtained from HiMedia Laboratories Pvt. Ltd. (India). Enzymatic analysis was done by plate assay method for different extracellular enzymes *viz.* Amylase, Catalase, Lipase and Protease (Panigrahi *et al.,* 2018).

Identification of Bacterial Isolate Using 16S rRNA Gene Sequence

The Phospho-endo bacterial isolate AP01 was identified at species level with 16s rRNA gene sequence homology method (Genexplore Diagnostics and Research Centre Pvt Ltd. Ahmadabad, Gujarat, India). DNA sequencing reaction of PCR amplicon was carried out with 8F & 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3500xl Genetic Analyzer. The 16S rDNA sequence was used to carry out BLAST (Zhang *et al.,* 2000) with the database of NCBI Genbank database website (http://www.ncbi.nih.gov). The phyllogenetic tree and the evolutionary history were conferred by using Neighbor Joining Method (Saitou and Nei, 1987) by using the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA 7) software (Kumar *et al.,* 2016). The analyses of number of base substitution per site from between sequence were conducted using Maximum Composite Likelihood model (Tamura *et al.,* 2004).

Effect of osmotic stress on the growth of the isolate AP01

To study the osmotic stress of the isolate AP01, different concentrations of PEG 6000 (0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% and 50%) was supplemented with nutrient broth and were inoculated with 2% inoculum of the isolate (AP01) and incubated at 37ºC for 48hr in an orbital shaker.

Growth of the isolate was determined spectrophotometrically at 600 nm.

Detremination of Thermal Death Point and Thermal Death Time of AP01

Thermal Death Point (TDP) and Thermal Death Time (TDT) of the isolate AP01 was determined by following the methods of Nadia *et al.,* (2018).

NaCl tolerance capacity of AP01

Different concentration of NaCl (0.5%, 1%, 1.5%, 3.0%, 5.0%, 7.0%, 8.0%, 9.0%, 10%, 12% and 15%) were taken in Nutrient Broth (NB) and inoculated with isolate (AP01) and incubated at 37±2°C for 24hours.

The absorbance of the cultures was measured using UV-Visible spectrophotometer (Systronics, 118, serial no. 2022) at 600 nm.

The viability of the isolate was tested by sub-culturing one loop of sample on Nutrient agar plate from the previously cultured test tubes.

Data analysis

All the experiments were carried out in triplicates. Error bars show Mean of triplicates with Standard Error of Mean. Two- way Analysis of variance (ANOVA) with the SPSS statistic program version 16.0 was performed for experiments.

Linear regression study was used to know the degree of association between pH and soluble phosphorus and among the variables taken for optimization of physical parameter studies i.e. Temperature, pH, Incubation day.

Results and Discussion

Isolation of endophytic bacteria

Four bacteria were isolated *in toto* on NA from different parts of *A. marmelos* isolated from petiole (AP01), leaf (AL01) and stem (AS01, AS02) with colonization frequency of (6.66%), (6.66%) and (13.33%) (Panigrahi *et al.,* 2018).

All the endophytic bacterial isolates were pure cultured on NA plates (3 streak method) and were preserved at 4°C on NA slants for future use.

Qualitative screening of phospho-endo bacterial isolate and media optimization

Of the four isolates, one isolate (AP01) showed a halo zone around the colony on PKVA agar medium indicating phosphate solubilizing ability of the isolate. While studying the phosphate solubilizing ability of the isolate on NBRIP and NBRIP-BPB agar medium, the highest PSI (4.5) was found on PKVA followed by NBRIP (3.3) and NBRIP-BPB (3.3) medium (Fig. 1).

Quantitative Estimation of soluble phosphorus and the drop in pH of different broth medium

This experiment was conducted to study the effect of broth medium on phosphate solubilizing ability of the isolate AP01. It was observed that the isolate in NBRIP medium could better able to solubilize the insoluble phosphorus $Ca_3(PO_4)_2$ as compared to PKV medium. While, the NBRIP medium was separately supplemented with $AlPO_4$ and $FePO_4$, as the source of insoluble phosphorous, we observed maximum solubilization of phosphorous in presence of $Ca_3(PO4)_2$ by the isolate.

The isolate could also able to solubilize the inorganic P source complexed in Fe and Al but in a declined manner (Fig. 2). Quantification was carried out on every 24h for a period of 11 days.

The phosphate solubilizing efficiency of the isolate AP01 increased from 1st day and maximum phosphate solubilization (70.200 µg/ml) was observed on 4th day with a drop down of pH(3.6) of the medium (Fig. 3A & 3B).

However, the amount of released phosphorus and pH of the medium remained constant after 4th day of incubation (Fig. 3C).

Optimization of growth parameters for maximum phosphate solubilization

From the growth parameters studies it was observed that the isolate solubilized maximum insoluble tricalcium phosphate in the NBRIP medium at 37º C as the optimal temperature [(70.403µg/ml) (Fig. 4A)] and with pH 8.0 (71.748µg/ml) as the optimal pH after which the solubilization decreased gradually (Fig. 4B). With above physical parameters, the isolate showed maximum phosphate solubilization when the medium was supplemented with 0.5% ammonium sulphate (79.76µg/ml) and 2% lactose (released P 87.283µg/ml) as optimal nitrogen (Fig. 4C) & carbon (Fig. 4D) source.

Identification of the isolate

The phosphate solubilizing endophytic bacterial isolate was identified by morphological, biochemical and molecular methods. The phenotypic characterization of the isolate, Gram's reaction, electron microscopic feature, biochemical characteristics, utilization of different sugars, TDP, TDT, Osmotic stress and NaCl tolerance capacities are presented in (Table 1). The isolate observed to be a gram-ve rod without spores.

The 16S rRNA gene sequence analysis of the phosphate solubilizing endophytic bacterial isolate AP01 showed 97% similarity with *Kosakonia cowanii* strain no. 888-76 with 99% query coverage which was confirmed with BLAST analysis data, thus the isolate AP01 was identified as *Kosakonia cowanii* strain and was submitted to GenBank with NCBI accession no. MK834804. The phyllogenetic tree for *Kosakonia cowanii* MK834804 was constructed by comparing the related nucleotide sequence based on maximum identity score from NCBI database (Fig. 5).

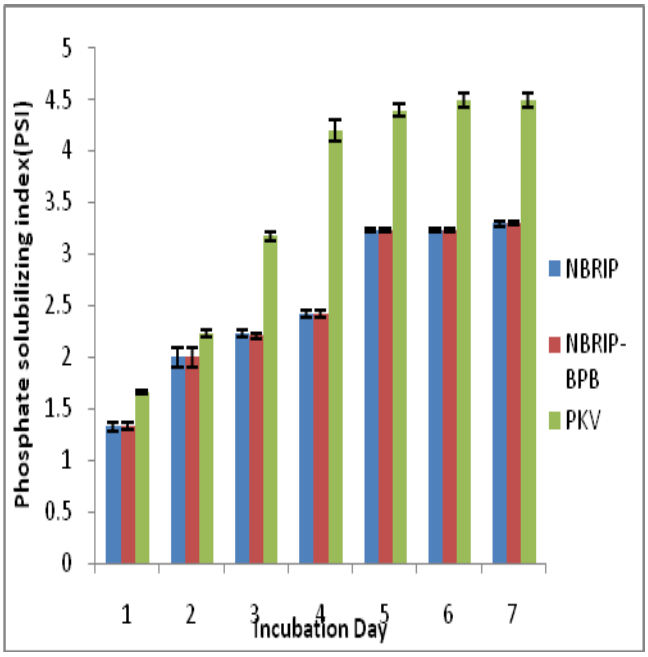


Fig. 1 PSI of AP01 on different media. Values are the mean of triplicates. Mean ± SD

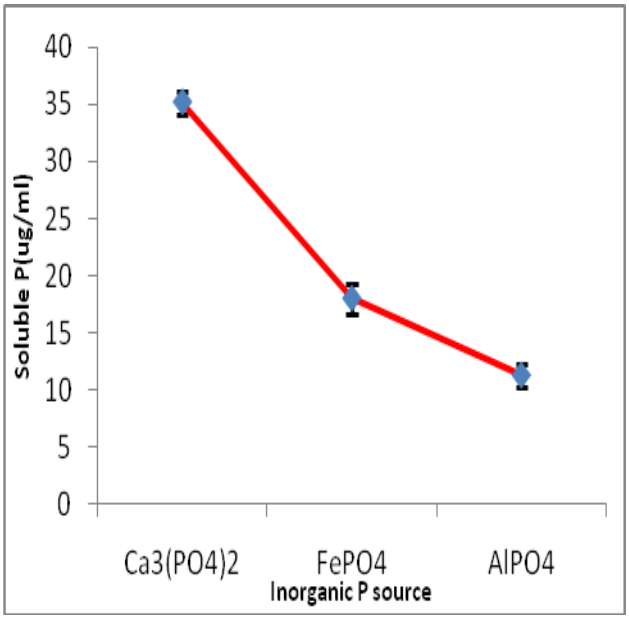


Fig. 2 Quantification of soluble phosphorus of different inorganic P source in NBRIP medium by the isolate AP01 after 48 hr of incubation. Values are the mean of triplicates. Mean ± SD

Fig.3 Determination of Optimal incubation period and pH for highest phosphate solubilization. A: Correlation between final pH and incubation day, Y = -0.095x+4.492 R^2=0.434; B: Correlation between soluble phosphorus and incubation day, Y=3.647x+37.55 R^2 = 0.539; C: Change in Phosphate solubilization and pH of the medium with incubation day

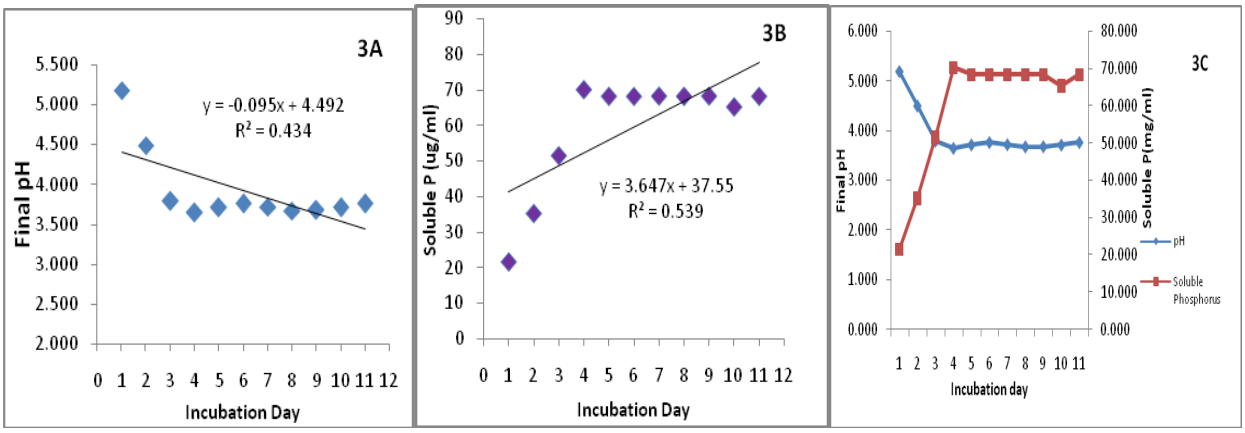
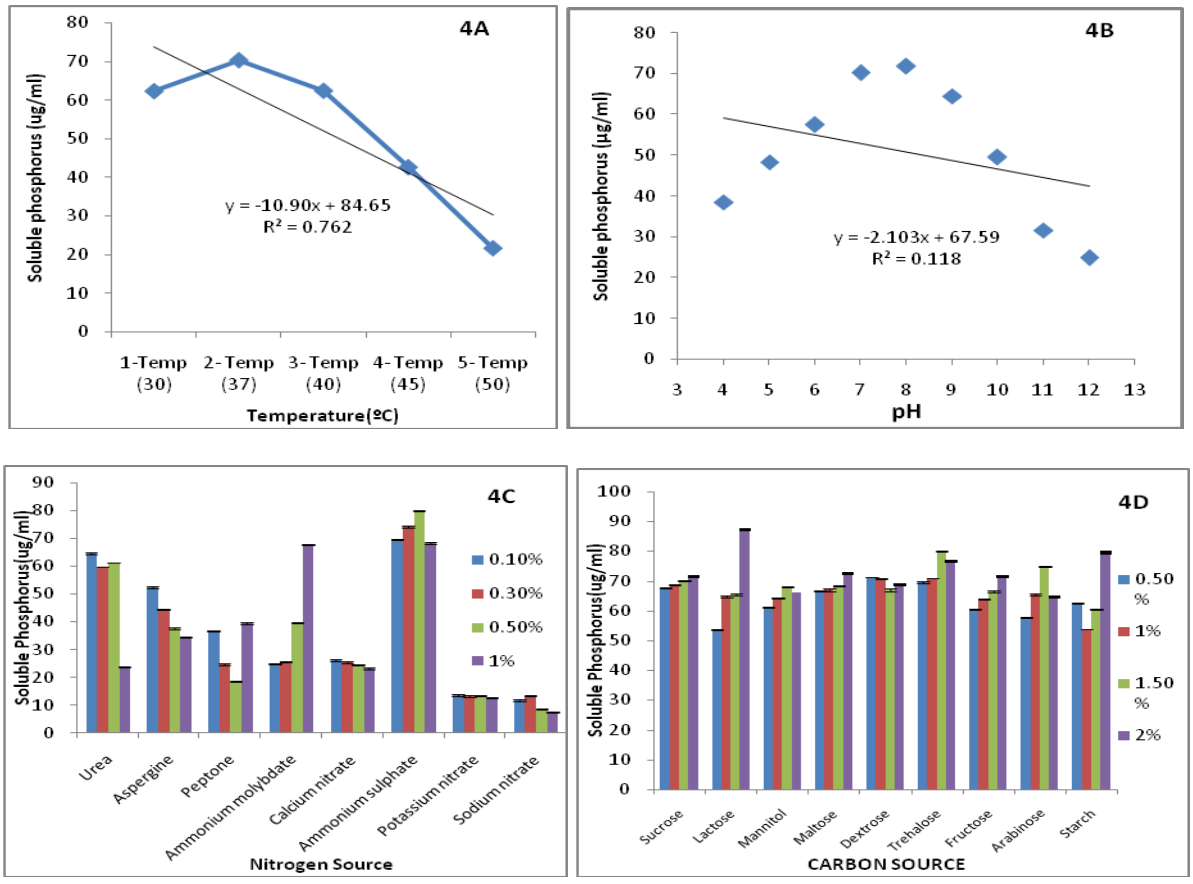


Fig.4 Effect of different growth parameters on phosphate solubilizing activity of AP01 A: temperature, B: pH, C: nitrogen source with varied concentration, D: carbon source with varied concentration. Values are the mean of triplicates. Mean ± SEM



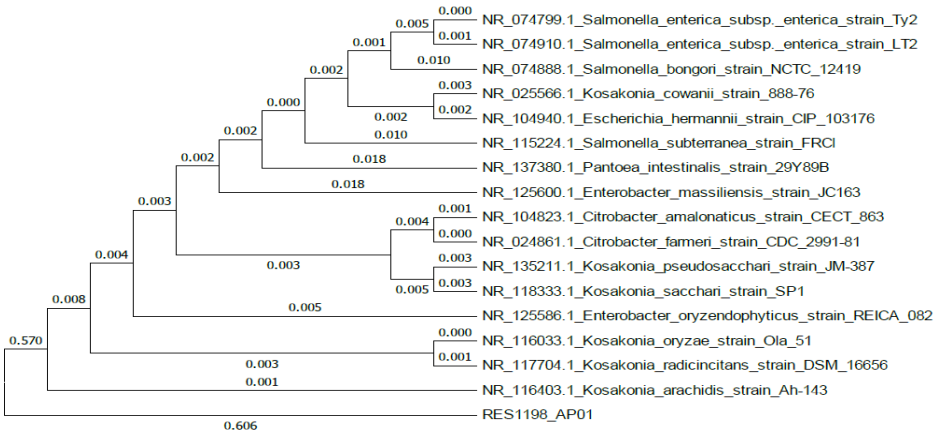


Fig. 5 The evolutionary history of the isolate AP01 was inferred using the Neighbor-Joining method. The optimal tree with the sum branch length= 1.29616796 is shown (next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were total of 846 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7.

Fig.6 HPLC chromatogram of the different organic acids present in the culture supernatant of isolate AP01, cultured in NBRIP medium with tricalcium phosphate at 37ºC, 4th day of incubation. The organic acids detected in the medium were oxalic acid (RT= 4.688), malic acid (RT= 5.445), tartaric acid (RT= 5.932) and gluconic acid (RT= 6.187)

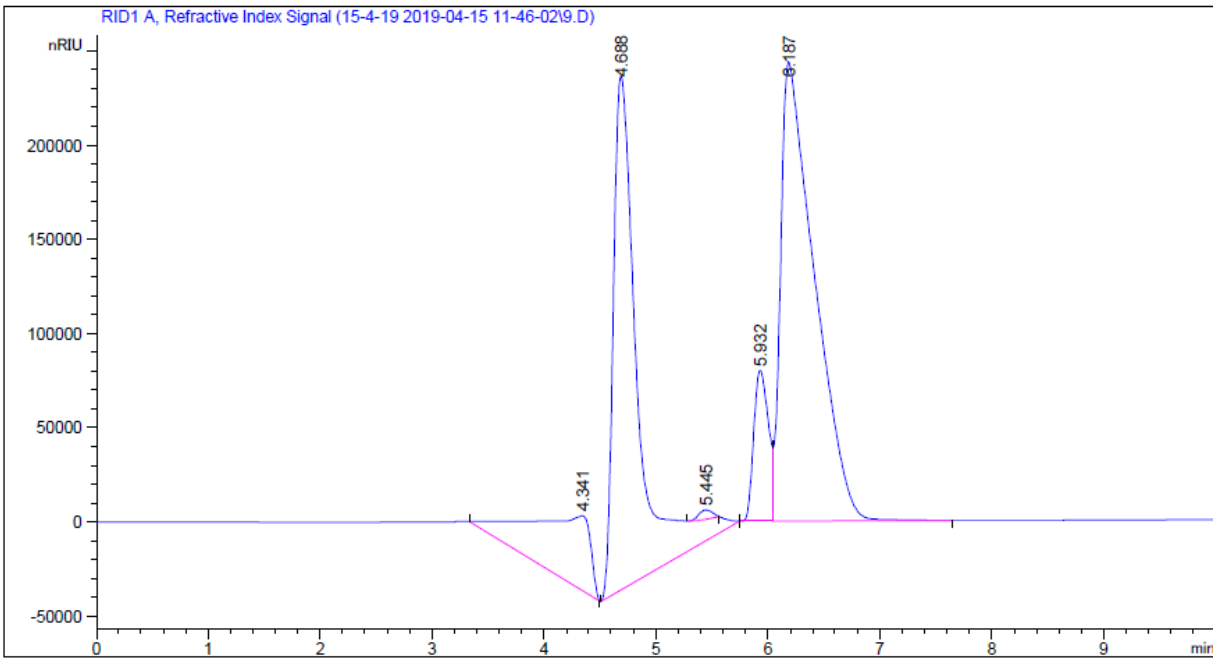


Table.1 Physiological and Biochemical characteristics of the isolate AP01

Characteristic	Findings	Characteristic	Findings
Morphology		Cellobiose	+
Colony morphology on Nutrient Agar	Medium, oval, Irregular, creamy, moist, opaque, measures about 2-4mm	Arabinose	+
MacConkey agar	Oval, whitish in colour, lactose fermenting	Melezitose	-
PKV agar	Thin, small, transparent	Sorbose	-
NBRIP agar	Oval, small	Glycerol	+
Shape	Rods	Salicin	+
Size(SEM Image)	0.6 x 1.1µm	Dulcitol	-
Gram's reaction	-ve	Inositol	+
Spores	-	Sorbitol	+
Biochemical Analysis		Mannitol	+
TSI test	+	Adonitol	-
Gas from D-glucose	+	Arabitol	-
H_2 S	+	Erythritol	-
Mannitol	+	Xylitol	-
Motility	+	α-Methyl-D-Mannoside	-
Indole	-	α-Methyl-D-Glucoside	-
Methyl Red	-	ONPG	+
Nitrate Reduction	+	Esculin	+
Simmon's Citrate test	+	Malonate	-
Urease test	-	Esculine Hydrolysis	+
		Ammonia production	+
Oxidase	-	**Physical Properties**	
VogesProskauer	+	NaCl tolerance (0-12%)	+
Sugar utilization		Thermal Death Point	57ºC
Glucose	+	Thermal Death Time	57ºC, 8 min
Lactose	+	Osmotic tolerance	25% PEG 6000
Sucrose	+	**Amino acid utilization test**	
Xylose	+	Ornithine utilization	-
Inulin	-	Lysine utilization	-
Sodium gluconate	-	Phenylalanine Deamination	-
Rhamnose	+	**Extracellular enzyme production**	
Saccharose	+	Amylase	+
Raffinose	+	Protease	-
Trehalose	+	Lipase	-
Melibiose	+	Catalase	+

+ Positive, - Negative, PKV Pikovskaya's agar media, NBRIP National Botanical Research Institute's phosphate growth mediumagar media.

Thus, from all the identification test studied above, the isolate AP01 was confirmed to be *Kosakonia cowanii*, a proteobacterium of family Enterobacteriaceae.

NaCl, Thermal and Osmotic stress tolerance of the bacterial isolate

It was observed that the isolate could tolerate 12% of NaCl (w/v) in the medium and Thermal Death Time of the isolate was observed to be 8 min at 57 °C respectively. From the Osmotic stress studies it was observed that the isolate AP01 could grow in presence of 25% of PEG 6000, but could not survive at 30% PEG 6000 in the medium. (Table 1).

Identification of organic acids produced by the isolate AP01

The identification of the organic acids in the culture medium of the isolate AP01 was carried through HPLC analysis on 4^{th} day of incubation. Four different organic acids viz. oxalic acid (RT= 4.688), malic acid (RT= 5.445), tartaric acid (RT= 5.932) and gluconic acid (RT= 6.187) were identified in the culture medium of the isolate AP01, by comparing the retention time with standard organic acids like tartaric acid, oxalic acid, malic acid, gluconic acid and citric acid (Fig. 6).

Endophytic bacteria as plant growth promoting agents have been reported by many authors proving its potential as IAA producer, phosphate solubilizer, iron chellator etc. in growth enhancement of different plants by different mechanism (Rashid *et al.*, 2012; Souza *et al.*, 2015). Literature stresses less exploitation of endophytes for their phosphate solubilizing capacity, the present experiment was conducted to evaluate the efficacy of endophytic bacteria from medicinal plant as phosphate solubilizers. The bacterial isolate AP01 was able to solubilize the insoluble phosphorus and was observed it as phospho-endo bacterium. While studying the potentiality of the isolate as phosphate solubilizer on different agar media like Pikovskaya, NBRIP, NBRIP-BPB, the isolate showed better result in Pikovskaya's agar media. Similar studies have been conducted by Hariprasad (2009), where isolates from tomato plant showed better results in Pikovskaya's solid medium, as observed in our investigation. But in contrast, the isolate showed higher degree of phosphate solubilization in NBRIP broth than in Pikovskaya's broth medium, during the quantitative assay in corroboration to Mehta *et al.*, (2000). Tricalcium phosphate ($Ca_3(PO4)_2$) was found to be the best inorganic P source followed by $FePO_4$ and $AlPO_4$ by the isolate AP01 to make avail of maximum soluble Phosphorus in the NBRIP medium and the same was also reported by Pradhan *et al.*, (2017).

Decrease in pH from neutral to pH 3.6 of the medium could be due to release of various organic acids during phosphate solubilization as reported earlier (Behera *et al.*, 2017 and Pande *et al.*, 2017). Statistically our result showed direct relationship between lowering of the pH and higher solubilization of phosphorus in the medium. From HPLC analysis four different organic acid *viz*. malic acid, tartaric acid, oxalic acid and gluconic acid were identified in the culture medium of the isolate AP01 which could be responsible for drop down of pH and solubilization of phosphate in the study. Vyas and Gulati, (2009) too reported secretion of malic acid, oxalic acid, gluconic acid, citric acid, succinic acid, formic acid in the culture filtrate of fluorescent *Pseudomonas* strains which corroborates with our observations. We recorded production of gluconic acid as the signature molecule produced by the phosphate solubilizing microbes of Enterobacteriaceae

family (Buch *et al.*, 2008; Chakdhar *et al.*, 2018). The isolate AP01 showed better phosphate solubilizing capabilities in NBRIP broth, at 37°C. In agreement with our study, Prasad (2014) reported that the suitable temperature for the growth condition and solubilization of phosphate by their isolate to be 37°C. The isolate showed highest solubilization of phosphate (70.200µg/ml) on 4th day of incubation after which the phosphate solubilization decreased. This could be attributable to the availability of soluble phosphorus in the medium which acts as an inhibitory effect on further solubilization (Chaiharn *et al.*, 2009) and also it could be due to depletion of nutrient in the medium (Kang *et al.*, 2002). On the other hand while studying the effect of C & N concentration on the phosphate solubilization it was observed that the isolate showed highest phosphate solubilizing activity in presence of 2% lactose (carbon source) and 0.5% ammonium sulphate (nitrogen source). In agreement to our investigation Rahman *et al.*, (2017) stated that ammonium sulphate is the best nitrogen source for bacterial strains when tricalcium phosphate is given as insoluble phosphorus source, but according to the same report fructose has highest effect on phosphobacteria *Pseudomonas & Bacillus* sp. for phosphate solubilization.

It is reported that, in terms of phosphate solubilizing efficiency, gram –ve bacteria are most promising rather than gram +ve bacterium Tripura *et al.*, (2007) which is in complete accordance with our study. On 16S rRNA sequencing studies the isolate was identified to be *Kosakonia cowanii* belonging to family Enterobacteriaceae. The bacterial isolates belonging to genera *Kosakonia*, *Pantoea* and *Bacillus* are the most promising phosphate solubilizer (Chakdhar *et al.*, 2018). In agreement with our conclusion, Chimwamurombe *et al.*, (2016) conducted an experiment in which, the endophytic phosphate solubilizing bacteria isolated from Marama seedling to be *Kosakonia sps.*

In conclusion, it can be told that the present study is of its kind on isolation of endophytic bacteria *Kosakonia cowanii* MK834804 from the medicinal plant *Aegle marmelos* with phosphate solubilizing ability. It is suggestive of that; the isolate could be used in agriculture as a novel source of phosphatic bio-fertilizer for enhancement of plant growth by making the insoluble P available to the plants with further investigations. Though it is a preliminary endeavor, studies such as this are a pre-requisite to exploit the biotechnological potential of endophytes (resident microbes inside the plants) for their use in agriculture.

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Compliance with ethical standards

Conflict of interest

The authors declare no conflict of interest.

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