



Identification of Phytase producing Bacteria C43 isolated from cattle shed soil samples of Hyderabad, A.P

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Abstract:

Phytases are the enzymes which hydrolyze phytic acid, a major storage form of phosphorus in cereals and legumes, to less phosphorylated *myo*-inositol derivatives releasing inorganic phosphate. The supplementation of animal feed with phytases reduces the cost of diets by removing or reducing the need for supplemental inorganic phosphate and increases the bioavailability of phosphorous in monogastric animals such as pig, poultry and fish which lack gastrointestinal phytases. These feed enzymes can also have a positive impact on the environment by allowing better use of natural resources and reducing pollution. Different soil samples - Rhizosphere soil, Cattle shed soil and poultry farm soil were collected from various regions of Hyderabad, Andhra Pradesh and were used as a source material for isolation and screening of phytase producing bacteria. Isolate C43 produced significantly higher phytase yield than other isolates and was chosen for species identification. Preliminary identification by microscopic and biochemical tests identified the isolate C43 as *Bacillus* sp. FAME analysis of the isolate by Gas Chromatography identified the isolate C43 as *Bacillus licheniformis*. Further, the identification of the strain was confirmed by subjecting it to 16S rRNA gene sequence analysis. The isolate C43 showed high similarity to *Bacillus subtilis* subsp. *spizizenii* strain 3EC5A1 and was thus designated as *Bacillus subtilis* C43.

Keywords:

Phytases, Phytic acid, FAME analysis, 16S rRNA, Gene sequence analysis.

Introduction:

Phytic acid (*myo*-inositol hexakisphosphate) is an anhydrous storage form of phosphate accounting for more than 80% of the total phosphorus in cereals and legumes [1, 2, 3]. Under normal physiological conditions phytic acid chelates essential minerals such as calcium, iron, zinc, magnesium, manganese, copper and molybdenum and preventing their absorption [4, 5, 6, 7, 8, 9]. The organically bound phosphate of phytic acid is not metabolized by monogastric animals such as pig, poultry and fish due

to lack of phytase and consequently contributes to the phosphorus pollution problems in areas of intensive livestock production [10, 11, 12, 13, 14]. The anti-nutritive properties and its value as possible phosphorus Source; have stimulated researchers to develop phytate hydrolysis methods. Phytases are the enzymes (*myo*-inositol hexakisphosphate phosphohydrolases) which hydrolyze phytic acid to less phosphorylated *myo*-inositol derivatives (in some cases to free *myo*-inositol), releasing inorganic phosphate [15]. The supplementation of animal feed with phytases reduces the cost of diets by removing or reducing the need for supplemental inorganic phosphate and increases the bioavailability of phosphorous in monogastric animals. Apart from contributing to improving nutritive value, these feed enzymes can also have a positive impact on the environment by allowing better use of natural resources and reducing pollution. These Phytases are widespread in nature and can be derived from a host of sources including plants, animals and microorganisms. Microbial sources are more promising for the production of phytases on a commercial scale [16, 17]. Some of the phytase producing microorganisms include bacteria such as *Bacillus subtilis* [18], *Escherichia coli* [19], Yeasts such as *Saccharomyces cerevisiae* [20], *Schwannomyces castelli* [21] and fungi such as *Aspergillus niger* [22], *A. oryzae* [23], *A. flavus* [24] and *Penicillium* sp. [25]. Due to several biological characteristics, such as substrate specificity, resistance to proteolysis and catalytic efficiency, bacterial phytases have considerable potential in commercial applications. The increasing potential of phytase application prompts screening for newer phytase producing microorganisms, which can meet the conditions favourable to the industrial production. Bacteria are though ubiquitous in their occurrence, the most common sources for their isolation are soils [26, 27].

The historical method for identification of unknown bacteria is dependent on the comparison of an accurate morphologic and phenotypic description of type strains with the accurate morphologic and phenotypic description of the isolate to be identified using standard references such as Bergey's Manual of Systematic Bacteriology. Frequently, there would be

no perfect match and a judgment would have to be made about the most probable identification which could vary among laboratories^[28]. A new standard for identifying bacteria was by comparing a stable part of the genetic code i.e. 16S rRNA gene^[29, 30, 31, 32, 33, 34, 35]. Thus, if the goal is to identify an unknown organism on the basis of no prior knowledge, the 16S rRNA gene sequence is an excellent and extensively used choice. Therefore, the present research study was taken up with the objective to isolate a potential phytase producing bacteria from various soil samples and identify the strain by studying morphological and biochemical characteristics and 16S rRNA gene sequence analysis.

Materials and Methods:

Isolation and screening of phytase producing bacteria:

Rhizosphere soil, Cattle shed soil and poultry farm soils were collected from various regions of Hyderabad, Andhra Pradesh. The samples were mixed in saline and the suspension was spread onto wheat bran extract agar plates^[18, 36]. The inoculated plates were incubated at 37°C for 1- 3 days and observed for the clear zones of hydrolysis around the colonies which gave an indication of extracellular phytase production. Further screening was done by subjecting the isolates to shake flask fermentation and assessing the enzyme activities. The enzymatic activity was measured by a modification of the Heinonen-Lahti method using sodium phytate as substrate^[37]

Identification and characterization of phytase producing bacterial isolate C43:

Morphological and Biochemical tests:

The isolate C43 was identified based on the identification scheme in Bergey's Manual of Systematic Bacteriology^[38]. The strain was initially examined for cell morphologies and cell arrangement by gram staining, presence or absence of spores and capsules and motility using microscopy. The various biochemical tests carried out were Indole test, Methyl red test, Voges proskauer test, Citrate utilisation tests, catalase, oxidase, urease, nitrate reduction, starch hydrolysis, gelatin hydrolysis, H₂S production and carbohydrate fermentation tests. The isolate was tested for fermentation of various sugars like Glucose, Lactose, Mannitol, Maltose, Sucrose, Xylose and Galactose. Various morphological and biochemical tests were carried out by the techniques described in the Mackie and Mc Cartney Practical Medical Microbiology^[39].

FAME analysis by Gas Chromatography:

FAME Analysis of the isolate C43 using Gas chromatography was carried out at Royal Life Sciences, Pvt. Ltd., Hyderabad, A. P. The bacterial cells from overnight culture were placed in a clean tube. 1.0ml of Reagent 1 (45g sodium hydroxide, 150ml methanol, and 150ml distilled water) was added to the tube and were sealed, vortexed briefly and heated in a boiling water bath for 30 minutes. The cooled tubes were uncapped, 2ml of Reagent 2 (325ml certified 6.0N hydrochloric acid and 275ml methyl alcohol) was added and briefly vortexed. After vortexing, the tubes were heated for 10 minutes at 80°C. The tubes were cooled and 1.25ml of Reagent 3 (200ml hexane and 200ml methyl tert-butyl ether) was added and gently tumbled on a clinical rotator for about 10 minutes. The tubes were uncapped and the aqueous (lower) phase was pipetted out and discarded. About 3ml of Reagent 4 (10.8g sodium hydroxide dissolved in 900ml distilled water) was added to the organic phase remaining in the tubes and tumbled for 5 minutes. About 2/3 of the organic phase was pipetted into a GC vial and subjected to analysis. The electronic signal from the GC detector was passed to the computer where the integration of peaks was performed. The electronic data was stored on the hard disk and the fatty acid methyl ester composition of the sample was compared to a stored database using the Sherlock pattern recognition software. The Sherlock MIS uses an external calibration standard developed and manufactured by Microbial ID, Inc.

16S rRNA gene sequence Analysis:

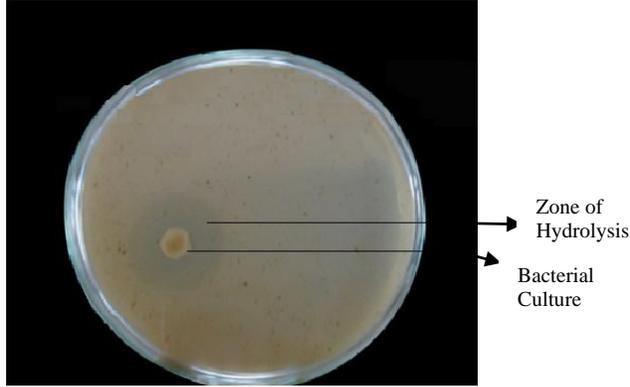
Sequencing of 16S ribosomal RNA was performed at RAS Life Sciences, Pvt. Ltd., Hyderabad, A.P. Bacterial genomic DNA was extracted from whole cells by using the standard method as described by Sambrook et al^[40]. PCR was performed to amplify a partial 16S rRNA fragment from the isolated DNA using universal primers 16F (5'CAGGCCTAACACATGCAAGTC3') and 1387 R (5'GGGCGGAGTGTACAAGGC3'). The sequences of the amplified 945 bp fragments were determined using an automated sequencer (Applied Biosystems). The 16S rRNA sequence from the isolated strain C43 was then aligned with reference sequences obtained from GenBank using BLAST

Results and Discussion:

In the present study, phytase producing bacteria were isolated from Rhizosphere soil, Cattle shed soil and poultry farm soil collected from various regions of Hyderabad, A.P., India. The focus of the work was to isolate bacterial species as producers of an extracellular phytase and identify the potential

phytase producing bacteria. The isolate C43 produced significantly ($P < 0.05$) higher yield than other isolates and this was chosen for identification and characterization studies (Figure 1).

Fig 1: Bacterial Isolate C43 on Wheat Bran Extract Agar



Preliminary identification of the isolate C43 was carried out by microscopic examination of morphological characteristics and biochemical tests (Table no: 1). The organism was seen as a motile, Gram-positive, sporulating rod. The organism showed Indole test negative, Methyl Red test negative, Voges Proskauer test positive and Citrate utilisation test positive. The organism is catalase and oxidase positive, reduces nitrate and hydrolyses starch and ferments various sugars like glucose, mannitol, maltose, sucrose, lactose and galactose with production of acid but no gas. Based on the identification scheme in “Bergey’s Manual of Systematic Bacteriology”, the isolate C43 was identified as *Bacillus* sp. Further the identification of the species was done by FAME Analysis using Gas chromatography. GC Analysis of fatty acid methyl esters was done by using MIDI Sherlock® Microbial Identification System, which was used for identification of the selected isolate. Based on the report, C43 was identified as *Bacillus licheniformis* with a similarity index of 0.869 (Figure 2).

However, these traditional methods of bacterial identification suffer from two major drawbacks. First, they can be used only for organisms that can be cultivated in vitro. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species. The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods [32, 41]. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. This method of "molecular identification" of the unknown bacteria involves determining the

DNA sequence of the 16S rRNA genes and comparing the generated sequence to a database of known sequences. Hence, the identification of the strain was further done by 16S rRNA gene sequencing analysis. The sequence of the amplified 945 bp fragment is given in Figure 3. Comparison of the 16S rRNA gene sequence of the isolate C43 with those in GenBank using BlastN showed 98% sequence homology with *Bacillus subtilis* subsp. *spizizenii* strain 3EC5A1. Therefore, it was designated as *Bacillus subtilis* C43. The sequence is deposited under accession no. KC243323 in GeneBank

Fig.3: 16S rRNA gene sequence of *Bacillus* sp. C43

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ATAATGCAGTCGAGCGAATGGATTAAGAGCT
TGCTCTTATGAAGTTAGCGGCGGACGGGTGA
GTAACACGTGGGTAACCTGCCATAAGACTG
GGATAACTCCGGGAAACCGGGGCTAATACCG
GATAACATTTTGAAGTGCATGGTTTCGAAATT
GAAAGGCGGCTTCGGCTGTCACCTTATGGATG
GACCCGCGTCGCATTAGCTAGTTGGTGAGGT
AACGGCTACCAAGGCAACGATGCGTAGCCG
ACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACG
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Fig. 2: GC – FAME analysis report

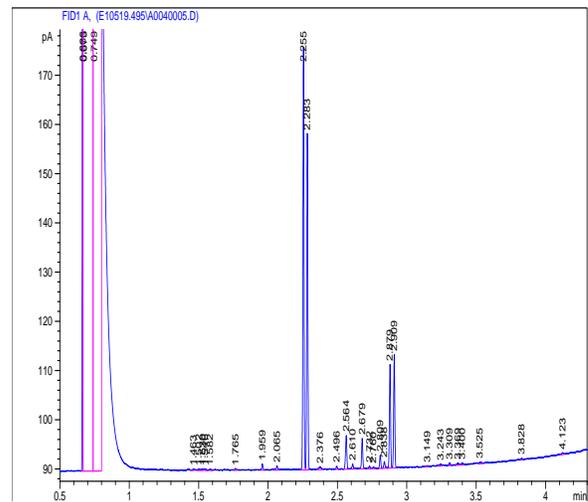


Table 1: Microscopic and Biochemical Characteristics of the Isolate C43

S. No	Identification Characteristics	Results
1	Morphology : a) Form b) Gram Staining c) Motility d) Spore e) Capsule	Rods Gram Positive Motile + -

S. No	Identification Characteristics	Results
2.	Biochemical Tests:	
	a)Indole Test	-
	b)Methyl Red Test	-
	c)Voges Proskauer Test	+
	d) Citrate Utilisation Test	+
	e) Nitrate Reduction Test	+
	f) Catalase Test	+
	g) Oxidase Test	+
	h)Starch Hydrolysis	+
	i) Gelatin Hydrolysis	-
	j)Urease test	-
k) H ₂ S Production	-	
3.	Carbohydrate Fermentation Tests:	
	Glucose Fermentation	A, NG
	Lactose Fermentation	A, NG
	Mannitol Fermentation	A, NG
	Maltose Fermentation	A, NG
	Sucrose Fermentation	A, NG
	Xylose Fermentation	NA, NG
Galactose Fermentation	A, NG	

Keywords:

- ✓ +: Positive
- ✓ -: Negative
- ✓ A : Acid production
- ✓ NA: No acid production
- ✓ G: Gas production
- ✓ NG:No gas producti

Conclusion:

Thus phytase producing bacteria were isolated from various soil sources and the high yielding bacterial isolate C43 was selected for characterization. Microscopic, Biochemical and 16S rRNA gene sequence studies revealed the newly isolated phytase producing bacterial isolate as *Bacillus subtilis*. In addition to phytase production, the isolate *B.subtilis* has other characters of commercial importance. The target protein is secreted to the culture medium which makes its purification simpler and more economical. Moreover, *B.subtilis* has a GRAS (Generally Regarded As Safe) status, allowing its use as feed additive. Further optimization studies will be made to develop cost effective phytase with improved properties to be used for the animal feed industries.

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