Isolation and biochemical characterization of a novel phytase producing bacteria Bacillus cereus isolate MTCC 10072

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Abstract

Bacillus cereus has been reported for the first time as phytase producer. Both qualitative and quantitative screening of the bacterial isolates was done to select potent phytase producer bacteria. Different biochemical tests, antimicrobial sensitivity tests and gram staining were performed on the selected strain for biochemical characterization of the organism. Out of 69 bacterial strains isolated from different sources NSD-Bacteria identified as Bacillus cereus MTCC 10072 from IMTECH Chandigarh was selected for further studies. Microscopic observations, biochemical test results and different enzyme assays showed the Bacillus cereus as a potent and novel phytase producer along with the production of other commercially important enzymes. Different experiments conducted in the present study will be useful for optimization of growth conditions for bacteria and thereby maximum enzyme production.

Keywords: Isolation, screening, biochemical characterization, phytase, Bacillus cereus

1. Introduction

Phytic acid and its salts, the phytates, contain the major part (approx. 90%) of the total phosphorus in the seeds of many plants e.g. cereals, leguminous plants etc. (Cheryan, 1980). Monogastric animals such as pig and poultry lack or have low phytase activities in their digestive tracts, so this form of phosphorus is not easily assimilated by them. This necessitates the addition to the forage/feed of sources of this element, for e.g. Ca₃(PO₄). So phytic acid is responsible for two problems in monogastric animals firstly, it acts as an anti nutritional factor since the phosphate moieties of phytic acid chelate essential minerals and their possibly binding to proteins. Secondly, the high level of undigested phytic acid in the faecal waste can be discharged in the sewage & become a primary cause of algal blooms in water environments (Dahiya et al., 2009). Because of the lack of adequate levels of phytases in monogastric animals, phytic acid is excreted in faecal waste, which is degraded by soil micro-organisms releasing phosphorus in the soil. The phosphorous reaches aquatic bodies and causes eutrophication (Singh and Satyanarayana, 2008). In order to overcome this problem, food, and feed can be supplemented with phytases for improving phosphorous bioavailability and reducing phosphorous excretion in the areas of intensive live stock populations. Phytase (myo-inositol hexakisphosphate 6-phosphorylase) are classified as the family of histidine acid phosphatases (Mitchell et al., 1997), which catalyze the hydrolysis of phytic acid (myo-inositol hexakisphosphate) to inorganic phosphate and myo-inositol phosphate derivatives in a stepwise manner. Present study mainly focuses on isolation, screening and biochemical characterization of a novel phytase producing micro organism.

2. Materials and Methods

2.1 Selection and screening of potent phytase producing bacteria
Sixty nine bacterial strains isolated from different soil and other samples, were quantitatively and qualitatively screened for phytase enzyme production. Quantitative screening was done on PSM (Phytase screening medium) screening medium with calcium phytate as substrate (Howson and Davis, 1983). Qualitative screening was done by determining the amount of inorganic phosphate liberated (Fiske and Subbarow, 1925) at 37 °C and pH 6.5±0.5. The phosphate released was determined spectrophotometrically according to Fiske and Subbarow (1925). One unit of phytase is defined as the amount of enzyme that liberates 1 nmol inorganic phosphate ml⁻¹ sec⁻¹ under standard assay conditions. Protein content was determined by Lowry’s method (Lowry et al., 1951) using bovine serum albumin as the protein standard.

2.2 Growth kinetics

Growth curve of the bacteria was plotted by growing the organism in nutrient broth. Growth was monitored by withdrawing the samples at desired time intervals and measuring the amount of biomass and inorganic phosphate released according to method described by Fiske and Subbarow (1925).

2.3 Other enzyme assays performed on the strain

Beside phytase production the bacteria was also tested for other enzymes like protease, lipases, amylase, xylanase and tannase.

2.4 Identification and Characterization

2.4.1 Colony morphology and Gram staining

The bacterial isolate was point inoculated on nutrient agar medium for 72h and observed under the microscope for colony morphological characteristics and gram staining.

2.4.2 Biochemical Tests

50μl of overnight grown bacterial culture was taken and inoculated in each biochemical test kit and was incubated overnight at 37 °C.

2.4.2.1 Carbohydrate analysis test

KB009 HICARBOHYDRATE™ kit is a standardized colorimetric identification system consisting of thirty five carbohydrate utilization tests. It is used to study the biochemical profile of selected strains and is based on the principal of pH change and substrate utilization. On incubation organisms undergo metabolic changes which were indicated by a spontaneous color change in the media that could be either interpreted visually or after addition of the reagent.

2.4.2.2 Analysis of bacterial strain for Gram-negative rod

KB002 HI Assorted™ Biochemical test kit is a standardized colorimetric identification system utilizing seven conventional biochemical tests and carbohydrate utilization tests. The test is based on the principal of pH change and substrate utilization.

2.4.2.3 Antibiotic susceptibility test

Selected bacterial strain was tested for antibiotic sensitivity with the help of 29 different antibiotic discs procured from Hi-media India. Then observation was done for sensitivity test.

3. Results and Discussion

3.1 Selection and screening of a novel bacterial strain producing high levels of phytase activity

Sixty nine different bacterial strains have been isolated from various samples collected from different geographical regions of India and were spread plated on nutrient agar media. The isolates were screened for the production of phytase by doing plate assay in the medium containing calcium phytate as the insoluble source of phosphorus. Many of the isolates except a few were able to grow on PSM agar plates, but only a few exhibited zone of calcium phytate hydrolysis. Those isolates, which gave a clear zone of hydrolysis, were further screened for phytase production in the medium containing phytic acid (sodium salt) as the sole source of phosphorus. On PSM agar plates containing sodium phytate, only a few bacterial culture displayed clear zone of phytate hydrolysis after double staining method (Bae et al., 1999). Reason behind this may be the secretion of organic acids like acetic acid, malic acid and others, which are known to solubilize calcium phytate and thus result in zone formation. Similar observation has been made by other phytase producing microorganisms (Casey and Walsh, 2004; Chadha et al., 2004). Based on
**Figure 4.1.** Screening of NSD-Bacteria (a) calcium phytate media (b) sodium phytate media

**Figure 4.2.** Growth curve of *Bacillus cereus*

**Figure 4.3.** Gram staining of NSD-Bacteria
Table 4.1 Observation table for zone of hydrolysis measured for the selected strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>0 day</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
<th>4th day</th>
<th>5th day</th>
<th>6th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus (NSD-Bacteria)</td>
<td>-</td>
<td>0.32 cm</td>
<td>1.8 cm</td>
<td>2.7 cm</td>
<td>4.0 cm</td>
<td>4.8 cm</td>
<td>4.8 cm</td>
</tr>
</tbody>
</table>

Table 4.2. Observation table for different enzyme assays

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Phytase</th>
<th>Amylase</th>
<th>Lipase</th>
<th>Xylanase</th>
<th>Protease</th>
<th>Siderophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSD-Bacteria</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

+ = Positive, ++ = moderately active, +++ = high activity, - = negative

Table 4.3. Microscopic observation for the characterization of NSD-Bacteria isolate

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colony morphology</th>
<th>Cell morphology</th>
<th>Gram reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSD-Bacteria</td>
<td>Cream, opaque, irregular margins, circular configuration, convex elevation, raised margins</td>
<td>Rod in shape, 2-4 µm in size, chain arrangement</td>
<td>Gram negative</td>
</tr>
</tbody>
</table>

Table 4.4. Screening for Carbohydrate Analysis Tests

<table>
<thead>
<tr>
<th>Observation</th>
<th>Biochemical agent(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Maltose, Fructose, Dextrose, Trehalose, Sucrose, Mannose, L-arabinose, Glycerol, Esculin, D-Arabinose, ONPG decarboxylase, Sorbose</td>
</tr>
</tbody>
</table>

Table 4.5. Screening for Gram-negative rods

<table>
<thead>
<tr>
<th>Observation(s)</th>
<th>Biochemical(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Citrate utilization, Lysine decarboxylase, Ornithine decarboxylase, Urease, Nitrate, Lactose</td>
</tr>
<tr>
<td>Negative</td>
<td>Phenylalanine deamination, H2S production, Adonital, Arabinose, Sorbitol</td>
</tr>
</tbody>
</table>

Table 4.6. Antibiotic susceptibility test

<table>
<thead>
<tr>
<th>Observation(s)</th>
<th>Antimicrobial agent(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly Sensitive</td>
<td>Amoxycillin, Ampicillin, Chlorotetracycline, kanamycin, PenicillinG, Rifampicin, Sulphomethizole, Tetracycline.</td>
</tr>
<tr>
<td>Resistant</td>
<td>ErythromycinE</td>
</tr>
</tbody>
</table>

High enzyme titers and screening results, two bacterial strains NSD-bacteria and NSB-10 were selected for identification. These strains were identified from IMTECH, Chandigarh as Bacillus cereus MTCC 10072 and Bacillus subtilis MTCC 10073 respectively. Out of these two identified strains NSD-Bacteria (Bacillus cereus MTCC 10072) was selected for subsequent studies.

3.2 Growth Kinetics

Bacillus cereus was found to have a lag phase of 27 h in nutrient broth medium. The log phase continued till 45 h followed by a stationary phase till 72 h and then the declining phase thereafter (Figure 4.2).

3.3 Production of different enzymes by NSD-Bacteria
On the basis of different enzyme assays performed on the NSD-Bacteria it showed positive results for phytase, protease, amylase and siderophore.

3.4. Identification and Biochemical Characterization

3.4.1 Microscopic studies

On the basis of microscopic observation and gram staining performed the bacterial isolate was identified as gram negative rod.

3.4.2 Biochemical analysis of selected strain

3.4.2.1 Carbohydrate analysis test

On the basis of experiments performed B. cereus showed positive carbohydrate fermentation results with maltose, fructose, dextrose, trehalose, sucrose, l-arabinose, mannose, glycerol, cellobiose, ONPG, esculin, D-trehalose, sucrose, l-arabinose, sorbose.

3.4.2.2 Analysis of Gram-negative rods

On the basis of experiments performed B. cereus showed positive characteristics for gram negative rods.

3.4.2.3 Antibiotic susceptibility test

NSD-Bacteria showed different level of sensitivity with different antibiotic discs procured form Hi-media India. Then observation was done on the basis of zone of hydrolysis produced in the presence of the selective antibiotic. Sensitivity ranges from highly sensitive to resistant.

4. Conclusion

Based on high enzyme titers and repeated screening NSD-Bacteria was found to be a potent and novel phytase producing bacteria. The strain was identified from IMTECH, Chandigarh as Bacillus cereus MTCC 10072. The selected strain showed positive results with double staining method confirming it as a phytase producer. Microscopic studies reveal a gram negative rod with 72 h of growth maxima as observed by growth kinetics. Along with phytase production NSD isolate showed positive results for siderophore, protease and amylase enzyme. Different biochemical tests performed with the bacteria will be used for optimization of growth conditions for maximum phytase production, selection of carbon source and sensitivity of the bacteria against different antibiotics.

References