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ARTICLE

Cow dung is an ideal fermentation medium for amylase production in solid-state fermentation by *Bacillus cereus*

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Abstract Amylase production by *Bacillus cereus* IND4 was investigated by solid state fermentation (SSF) using cow dung substrate. The SSF conditions were optimized by using one-variable-at-a-time approach and two level full factorial design. Two level full factorial design demonstrated that moisture, pH, fructose, yeast extract and ammonium sulphate have significantly influenced enzyme production ($p < 0.05$). A central composite design was employed to investigate the optimum concentration of these variables affecting amylase production. Maximal amylase production of 464 units/ml of enzyme was observed in the presence of 100% moisture, 0.1% fructose and 0.01% ammonium sulphate. The enzyme production increased three fold compared to the original medium. The optimum pH and temperature for the activity of amylase were found to be 8.0 and 50 °C, respectively. This enzyme was highly stable at wide pH range (7.0–9.0) and showed 32% enzyme activity after initial denaturation at 50 °C for 1 h. This is the first detailed report on the production of amylase by microorganisms using cow dung as the low cost medium.

1. Introduction

Amylases are the most important industrial enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25–30% of the world enzyme market [1]. These enzymes have a great commercial value in biotechnological applications ranging from food, fermentation, textile to paper industries [8]. Submerged fermentation (SmF) is generally used for the production of enzymes including amylases. However, Solid-state fermentation (SSF) replaces SmF as it mimics the natural habitat of microorganisms. SSF is a better choice over SmF due to its simplicity, low capital investment, lower energy requirement, less water output, and lack of foam built up [4,12].

Agrowastes like wheat bran, rice bran, and coconut oil bran have replaced the high cost media generally used in submerged fermentation for amylase preparation because of their simplicity, low cost, easy availability, and lesser water output. Additionally it solves the pollution problem occurring due to their disposal in the surrounding [19]. Recently, various agrowastes

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were used as the substrates for the production of amylases in SSF. The organisms namely, *Bacillus amyloliquefaciens* (MTCC 1270) [18], *Anoxybacillus flavithermus* sp. [11], *Bacillus licheniformis* ZB-05 [7], *B. amyloliquefaciens* P-001 [5], *Bacillus subtilis* (MTCC 121) [17] and *B. licheniformis* RT7PE1 [21] were used for the production of amylases and enzyme properties were studied. Recently, cow dung was used as the substrate for the production of proteolytic enzymes [23,24]. However, reports on the utilization of cow dung for the production of amylase may be little or perhaps nil. For the maximum enzyme production, medium optimization is a first step for its commercial usage. The present work describes the effects of culture conditions on amylase production in SSF using cow dung substrate and the properties of enzyme by *Bacillus cereus* IND4. Optimal culture conditions and fermentation parameters were assessed by using one variable at a time method followed by 2^5 full factorial design and CCD. The amylase enzyme was partially characterized for various industrial applications.

2. Materials and methods

2.1. Microorganism

Around 0.1 g of fermented rice was transferred to an Erlenmeyer flask (100 ml) with 50 mL of sterile double-distilled water, shaken for 20 min, and 1 ml of this solution was resuspended in sterile double-distilled water and aliquots were then spread on nutrient agar plates composed of the following (g/L): peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5 and sodium chloride, 5.0 (pH 7.0). Twelve organisms were isolated and the isolated organisms were screened for amylase activity. The organisms were grown on nutrient agar plate containing 1% soluble starch. After 24 h incubation at 37 °C, 1% iodine was poured on the starch agar plates. A clear zone of hydrolysis around the colony indicates a positive result.

2.2. Identification of the amylase enzyme-secreting organism

The isolated strain IND4 with highest activity was identified on the basis of the biochemical properties, the phenotypical characteristics, and the 16S rRNA gene sequencing. The genomic DNA was extracted from the cells of an 18-h cultured IND4 strain by using a QIAGEN DNA purification kit (Germany) according to the manufacturer's instructions. The 16S rRNA gene of the isolate was amplified by polymerase chain reaction (PCR) using the upstream primer P1: 5′-AGA GTTTGATCMTGGCTAG-3′ and the downstream primer P2: 5′-ACGGGCGG TGTGTRC-3′ (Sigma–Aldrich). Amplification of DNA was carried out using the research gradient Peltier Thermal cycler machine PTC-225 and a DNA polymerase (Sigma) under the following conditions: denaturation at 95 °C for 3 min followed by 30 cycles at 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min and 50 s. The amplified product was sequenced at Scigenome Laboratories, India. Sequence comparison with databases was performed using BLAST through the NCBI server. The isolate IND4 was identified as *B. cereus* IND4. The sequence was submitted to the GenBank database, and an accession number was assigned. The GenBank accession number of the sequence reported in this article is KF250420.

2.3. Solid state fermentation

Cow dung was obtained from the farm house. It was dried for 7 days, powdered and used as the substrate. Fermentation was carried out in Erlenmeyer flasks (100 ml) with 2.0 gm cow dung substrate, supplemented with carbon source (1%), nitrogen source (1%) and inorganic ion (0.1%). The pH of the medium was adjusted using 0.1 M buffer at various pH range (6.0 to 10.0). Moisture of the medium was adjusted to 100% and autoclaved for 121 °C for 20 min. During the preliminary screening process, the experiments were carried out for 96 h and it was found that after 72 h, maximum enzyme production occurs. Hence, all experiments were carried out for 72 h.

2.4. Enzyme extraction and assay

The fermented substrate was mixed thoroughly with 20 ml of sterile distilled water and placed in an orbital shaker at 150 rpm for 30 min. After this, it was centrifuged at $10{,}000\times g$ for 10 min, and the supernatant was used as the crude enzyme. The amylase enzyme was assayed accordingly to the method described by Miller [9] using the UV–visible spectrophotometer (Eltek, India). One unit of amylase activity was defined as the amount of enzyme that releases 1 μg of reducing sugar as glucose per ml per min under the assay conditions.

2.5. Statistical optimization of amylase production by *B. cereus IND4*

In this study, maximum amylase production by *B. cereus* IND4 was attained by response surface statistical optimization methods employing different process parameters under SSF. Significance of various medium constituents towards amylase production was tested initially by a full factorial experimental design (FFD). The factors and ranges were selected by one-factor experiments (data not shown). The 2^5 full factorial design consisted of a set of 32 experimental runs in which the selected five factors (moisture, pH, fructose, yeast extract and ammonium sulphate) were kept either at their high (+) or low (−) levels to find out the most significant factors on amylase production. Table 1a lists the variables and levels in detail. All these experiments were carried out in 100 ml Erlenmeyer flasks containing 2.0 gm of production medium (cow dung) with appropriate media components.

The 2^5 factorial design was based on the following first-order polynomial model:

Table 1a Independent variables and their levels for the 2^5 factorial experimental design.

Symbol	Variable name	Units	Coded levels	
			−1	+1
A	Moisture	%	80	100
B	pH		7	8
C	Fructose	%	0.1	1
D	Y. extract	%	0.1	1
E	A. sulphate	%	0.1	0.5