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Original Research Article

Isolation and screening of agro-waste substrates for protease production through solid state fermentation

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A B S T R A C T

Soil samples from different locations like cultivated soil, garden soil and compost were screened for the presence of proteolytic microorganisms which were capable of producing proteases. A total of 18 soil samples were screened to get 33 isolates amongst them 10 strains showed proteolytic activity. The isolate MSN33 showing larger zone of hydrolysis on skimmed milk agar was chosen for further studies. The organism was tentatively identified as gram negative bacillus and was subjected to 16S rRNA sequencing which revealed the organism to be *Acinetobacter calcoaceticus*. For the maximum production of proteases different agro-based substrates were tested through solid state fermentation as they are inexpensive and are used only as animal feed. The substrates chosen for the studies were wheat bran, green gram husk, fried gram husk and cattle feed to check their efficacy in protease production. Amongst the substrates tested cattle feed emerged as the best substrate showing maximum protease production of 8.5 IU. This was then used for further optimization studies.

Keywords

Teases;
soil isolate;
agro-waste substrates;
cattle feed.

Introduction

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. All enzymes which have been purified are protein in nature, and may or may not possess a non-protein prosthetic group (Underkofler, *et al.,* 1958) Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine

chemical industries. Currently, enzymes are becoming increasing important in sustainable technology and green chemistry (Thomas and Raja, 2005).

Proteases constitute a class of industrial enzymes, which alone form approximately 60% of the total world-wide enzyme production (Chu 2007). Protease are the class of enzymes, which occupy key position with respect to their applications in both physiological and commercial fields (Godfrey and West,1996). Among all the proteases, alkaline proteases are robust in nature and are primarily used as detergent additives (Gupta *et al*., 2002a). Proteases represent one of the three largest groups of industrial enzymes and account for about 60% total worldwide sale of enzymes (Rao *et al.,* 1998). Several microbial strains including fungi (*Aspergillus flavus, Aspergillus miller*, *Aspergillus niger* and *Penicillium griseofulvin*) and Bacterial (*Bacillus licheniformis, Bacillus firmus, Bacillus alcalo Bacillus subtilis* and *Bacillus thuringiensis*) have reported to produce proteases. Among the various proteases, microbial proteases play an important role in biotechnological processes. Nowadays, the overall cost of enzyme production is very high as the cost of substrate and medium used is very expensive therefore; development of novel processes to increase the yield of proteases with increasing the production cost is highly appreciable from the commercial view point. To achieve these objectives, during the recent years, efforts have been directed to expose to reduce the protease production costs through improving the yield, and the use of either cost free of low cost feed stocks or agricultural by products as substrates for protease production. Production of these biomolecules using agro-based substrate under solid state fermentation and conditions provide several advantages in producing, cost effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization, etc. (Pandey *et al*.,2000).

Alkaline proteases produced are of special interest as they could be used in manufacture of detergents, food, pharmaceuticals and leather (Saeki et al. 2007, Dias et al. 2008). In recent times a many studies have been conducted to characterize alkaline protease from different microorganisms. However, many of the alkaline proteases applied to industrial purposes face some limitations such as low stability towards surfactants and production cost of the enzymes arisen from growth medium (Joo and Chang 2005). It is known that the amount of enzyme produced greatly depend on strain and growth conditions. Using of cost effective growth medium for the production of alkaline proteases from diverse microorganisms. Therefore, there is a need to the search of new strains of bacteria that produce proteolytic enzymes with novel properties and the development of low cost media. Many different bacterial, fungal and actinomycete strains are involved in the production of proteases.

Among microbial communities involved in different ecosystems such as soil, freshwater, wastewater and solid wastes, several strains belonging to the genus Acinetobacter have been attracting growing interest from environmental and a biotechnological point of view. Bacteria of this genus are known to be involved in biodegradation, leaching and removal of several organic and inorganic man-made hazardous wastes.

Acinetobacter strains are also well represented among fermentable bacteria for the production of a number of extra and intracellular economic products such as lipases, proteases, cyanophycine, bioemulsifiers and several kinds of biopolymers (Desouky Abdel-El-Haleem 2003).

The focus of this study therefore, was concentrated on the isolation and screening of potential protease producing strains and selection of agro-waste substrates for maximum protease production.

Materials and Methods

Sample collection and isolation of strains

Soil samples were collected from different locations like cultivated soil, garden soil and compost soil in sterile polyethylene bags and refrigerated till use. 1g of the soil sample was aseptically added to 9 mL sterile distilled water and shaken on a gyratory shaker for 30 min. Serial dilutions (up to 10-6) of suspensions were prepared and 0.1 mL of aliquots were aseptically spread on Starch Casein Agar with the following composition, Starch 10.0, Casein 0.3, Na Cl 2.0, K_2HPO_4 2.0, $CaCO_3$ 0.02, $MgSO_4.7H_2O$ 0.05, $FeSO_4.7H_2O$ 0.01 and incubated at 37°C for 24 h, well isolated colonies were picked and then plated on skim milk agar plates containing peptone (0.1%), NaCl (0.5%) and skim milk (10%) supplemented with cycloheximide (50 µg/mL).

In situ protease production was demonstrated by the clearing of opaque milk proteins in the surrounding of colonies growing on the plates. The high-yield protease strains were evaluated by measuring the zone of hydrolysis on the skimmed milk agar (Plate 2.1).

Phenotypic identification

The best isolate was identified by basic morphological and biochemical parameters. The colony characteristics, cell morphology, Gram-staining reaction, nutritional susceptibility, and other biochemical characteristics of the isolate were studied for identification at the genus level (Table-2.2). The isolate was then subjected to 16SrRNA sequencing for species level identification.

Molecular identification

The phenotypically identified genus producing maximum extra cellular protease activity was further analyzed by 16S rRNA amplification and nucleotide sequencing. The sequence after amplification was evaluated with bioinformatics tools to identify the isolate upto the species level. This work was outsourced, and carried out by Bhat Biotech India (P) Ltd., Bangalore.

Screening of agro-waste substrates

In order to produce higher yield of alkali ne protease through solid state fermentation various agro-waste substrates having sufficient amount of nutrients have been chosen. In all 5 different substrates were screened for the production proteases by solid state fermentation. Wheat bran, Green gram husk, Bengal gram husk, tapioca powder and cattle feed. All the above mentioned substrates were procured from local market. Plate

Preparation of Substrates for SSF

Various agro-based substrates were screened for the production of alkaline protease through solid-state fermentation (SSF). Twenty grams of each of the above substrates (wheat bran, fried gram husk, green gram husk, tapioca powder and cattle feed) were taken in 250ml Erlenmeyer flask and rehydrated to 65% of moisture content by adding sterile distilled water. The cotton plugged flasks were autoclaved at 121^{o}C for 15 min and allowed to cool at room temperature. The contents of the flasks were inoculated with 1ml of inoculum. The flasks were mixed thoroughly by gently beating on the palm of the hand and thus prepared flasks were kept in slanting position and incubated at 37^{o}C (Lingappa *et al.*, 2004).

Enzyme extraction

After the desired incubation, a known quantity (1 g) of the fermented material was mixed with10 ml of 0.1M phosphate buffer and homogenized by shaking for 30 min and filtered throughcheese cloth. Cell free supernatant was obtained by centrifuging the extract at 10,000 x g for 30min and filtering through Whatmann filter paper. The volume of filtrate which contained the crude enzyme was measured and used for the protease assay.

Assay for proteolytic activity

The protease was assayed as per Chi et al., (2007) using casein as a substrate. One unit of enzyme activity (U) was taken as the amount of enzyme liberating 1µg of tyrosine per ml per minute under the assay condition. The estimation of enzyme activity was based on the tyrosine standard calibration curve.

Results and Discussion

Screening of microorganisms

A total of 33 isolates were screened out of which 10 isolates showed proteolytic activity. Amongst them a gram negative bacterium showing greater zone of casein hydrolysis on skimmed milk agar was isolated and named tentatively as MNS33 which exhibited the following colony morphology, opaque, circular and convex surface, creamish white pigmentation. The bacterium showed a zone of 3 mm in diameter which adhered to the agar surface (Plate 1). The difference in the zone of hydrolysis between the different isolates may be attributed to the screening conditions and their proteolytic capacities. Hence the strain MNS33 was chosen for further maximum protease production studies.

Morphology and biochemical characteristics

The results of biochemical characterization for the isolate MNS33 closely matches with the common characters of the organism *Acinetobacter* sp. and that was also confirmed from various morphological features and biochemical tests (Table 1). In addition, the isolate showing catalase positive and oxidase negative confirms the genera. According to the procedure for determination of phenotypic properties recommended by the international committee on systematic bacteriology (ICSB), the isolate was tested for acid production from glucose and lactose which showed positive.

The isolate also showed positive for acid production from xylose and exhibits few other characters of genera *Nisseria* and

*Moraxella***.** Moreover the isolate showing positive for starch and casein hydrolysis, negative for gelatin demonstrates that it possesses variant characters of the genera *Acinetobacter*.

Molecular taxonomy

Molecular analysis of 16S rRNA amplification for the isolate was carried out by Bhat Biotech (P) Ltd, Bangalore and it provided the nucleotide sequence of 1318bp units. Sequences were compared to the non-redundant NCBI database by using BLASTN, with the default settings used to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbours was aligned using CLUSTAL W2 for multiple alignments with the default settings. The multiple-alignment file was then used to create phylogram using MEGA5 software.

Screening of substrates

In solid state fermentation the substrates serve as means of anchorage to the microorganism. It also provided good substrate porosity for growth. It becomes imperative to select the suitable solid substrate for good growth and maximum product formation. Of the different substrates tested for growth and protease production by *Acinetobacter calcoaceticus* wheat bran, bengal gram husk, green gram husk, tapioca powder and cattle feed (Fig: 2.2) all the substrates supported the growth of the organism and protease production except for tapioca powder, this may due to the absence of nutrients in case of tapioca powder which could bring about protease production.

In this study cattle feed supported good protease production hence was used as substrate for further optimization studies (data not shown). The maximum protease activity achieved may be due to the growth and their tolerance to low water activity and high osmotic pressure conditions which makes the *Acinetobacter calcoaceticus* efficient for bioconversion of solid substrates.

Industries involved in microbial technology are involved in setting up new bioprocesses. Many companies worldwide are investing in newer ventures for microbial products. The need of the hour is to explore microbial strains with novel traits. In recent years there has been a phenomenal increase in the use of proteases as industrial enzymes. For industrial production of proteases, synthetic media are usually employed that contain very high concentration of carbohydrates, proteins, and other media components. In this context studies were aimed at overproduction of proteases from agrowaste substrates would serve in reducing the costs by designing newer bioprocesses.

From our study we could successfully isolate a gram negative bacteria, *Acinetobacter calcoaceticus* identified through biochemical studies and 16s rRNA sequencing capable of producing proteases from agrowaste substrate like cattle feed. The reports on the use of this isolate for production is very scanty, suggesting it to be a novel strain for protease production, thus paving way for the utilization of this strain and the substrate for the industrial production of proteases. Optimization studies for the maximum production of the protease enzyme can be taken up further.

Plate.2.1 Zone of clearence on skimmed milk agar

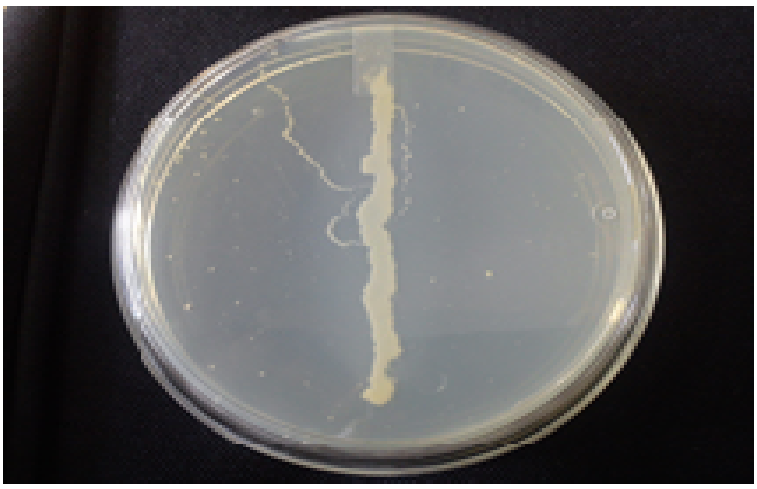


Table.2.2 Biochemical characteristics of Acinetobacter sp.

Test Organism	**Acinetobacter spp.**
Morphology	Gram negative
Motility	Non motile
Catalase	+
Oxidase	+
MacConkey agar	+
Acid from: Glucose	+
Xylose	+
Mannitol	-
Sucrose	+
Galactose	+
Mannose	+
Rhamnose	-
Lactose	-
Maltose	-
TSI Acid:	
Slant	+
Butt	-
H2S	-
Citrate	+
Urea, Christiansen	-
Nitrate	-
Methyl red	-
Voges Prausker	-

Table.2.3 Phylogenetic Tree of the organism

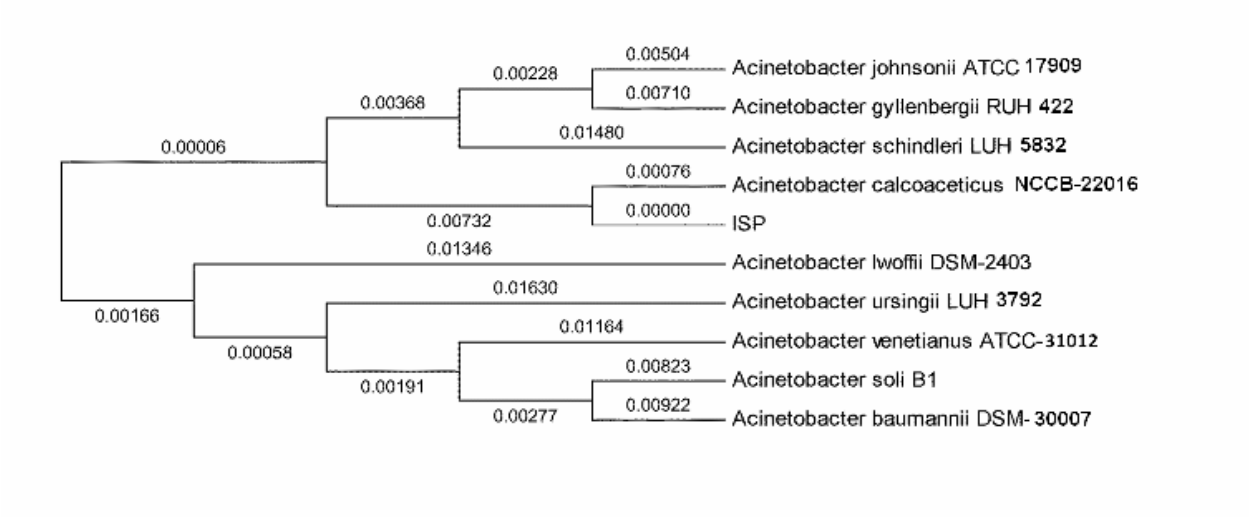


Plate.3.5 Different Agro-wastes

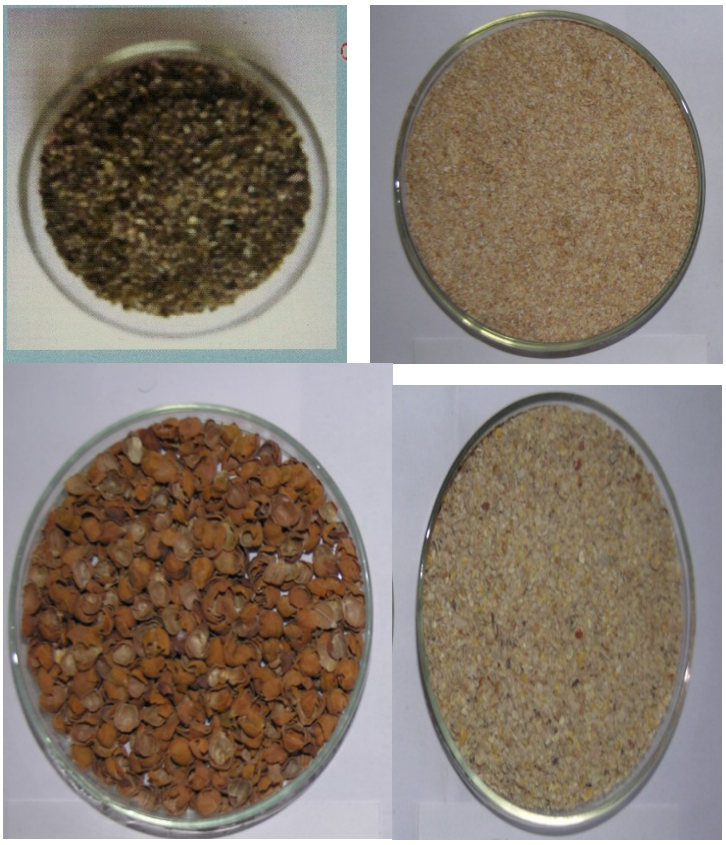
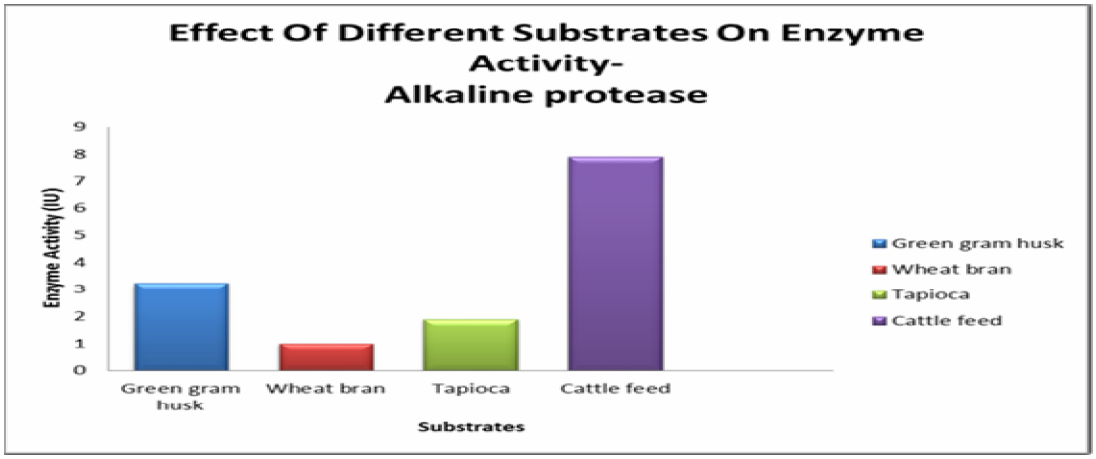


Fig.2.2 Graph showing protease production with different substrates



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