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RESEARCH ARTICLE

Isolation and Screening for Protease-Producing *Bacillus* Species from Soils in Awka Anambra State South Eastern Nigeria

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ABSTRACT

Isolation and screening for protease producing *Bacillus* species from distinct soils in Awka Anambra state were studied. Soil samples were collected from different locations and 1g of each of them was added to 9ml of sterile distilled water. Thereafter the sample was shaken and heated at 90^0C for 15min and then plated on nutrient agar medium. *Bacillus* species were isolated from all the soils sampled and were identified using standard methods. A total of 54 *Bacillus* species was isolated with the soil at Amaenyi having the highest of 15, while the soil at Ifite Awka recorded the lowest of 5. The *Bacillus* species identified included *B. licheniformis, B. subtilis, B. megaterium, B. stearothermophilus, B. macerans, B. coagulans, B. pumilus* and atypical strains. *B. licheniformis* recorded the highest percentage occurrence of 24.1%, while atypical strains recorded the least percentage occurrence of 5.56%. *Bacillus megaterium, B. macerans* and *B.coagulans* had a percentage occurrence of 9.26%. Twenty three selected *Bacillus* species screened for protease production exhibited varying ability to produce protease. The highest zone of clearance was observed in *Bacillus subtilis* SE2 (12.3mm), *B. subtilis* SU8 (10.8mm) and *B. licheniformis* SE9 (10.2mm), while *Bacillus macerans* produced the lowest zone of clearance of 4.3mm.The highest protease production of 102 and 133 U/ml by *Bacillus subtilis* SE2 and *B. subtilis* SU8 was recorded after 48h, while *B. licheniformis* SE9 accumulated the highest protease yield of 148 U/ml after 72h of incubation. The result of the study showed that protease - producing *Bacillus* species was present in the soil.

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INTRODUCTION

Enzymes are protein biocatalyst produced by living cells that facilitates metabolic reactions without themselves being altered after the reaction (Talaro and Talaro, 2002). They are derived from a variety of plants, animals and microorganism. The production of microbial enzymes for commercial exploitation has been on since the emergence of industrial microbiology (Burham *et al*., 2003). Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce and their enzyme content is more predictable, controllable and reliable (Burhan *et al*., 2003).

Bacillus species produce a large variety of extracellular enzymes, such as proteases which have significant industrial importance. These proteases catalyse the cleavage of peptide bonds in proteins. They constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme sales (Nascimento and Martins, 2004; Beg and Gupta, 2003; Ellaiah *et al*., 2003).

Proteases are important enzymes employed in the leather industry for bioprocessing of hides particularly for the dehairing and bating processes (Taylor *et al*., 1987). Also extracellular proteases can be used in the treatment of wastes such as industrial effluent, waste water and domestic sewage, since a large portion of dissolved organic matter and particulate organic matter in such wastes consist of proteinaceous material (Chost *et al*., 1986). They are also employed in food, pharmaceutical, detergent and brewing industries (Pastor *et al*., 2001; Ward, 1985).

The objective of this study was to isolate *Bacillus* species, screen them for proteolytic activity and grow them in submerged culture.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from a depth of 5cm from 6 locations in Awka town which included Amaenyi, Government house, Amawbia, Agu Awka, Ifite Awka and Eke Awka. They were transferred into polythene bags and taken to the laboratory for analysis

Isolation of *Bacillus* species from soil

One gram of soil was added to 9ml of sterile distilled water contained in a test tube and shaken properly. The content of the test tube was heated thereafter at 90^0C for 15min and cooled. After 6 fold serial dilution, aliquots of 0.1ml was plated in duplicates on nutrient agar plates and incubated at 30^0C for 24h. Pure culture was made from each distinct colony that developed and stored in a refrigerator at 4^0C for cultural and biochemical identification. The method of Gordon *et al*. (1973) was used for the identification of isolates.

Screening of *Bacillus* species for protease production

A loopful of each of the randomly selected *Bacillus* species was inoculated on a nutrient broth and incubated at 30^0C for 24h. Aliquots of 0.1ml (1.7×10^4 Cfu/ml) of each of them was taken and plated in duplicates on skim milk agar plates and incubated at 35^0C for 48h. The production of protease by the organism was indicated by zone of clearing around the colonies growing on the agar plates.

Submerged production of protease

The 3 highest protease producers were selected for the submerged production of the enzyme. A loopful of each of the randomly selected *Bacillus* species was inoculated on a nutrient broth and incubated at 30^0C for 24h. Two(2) ml suspension of each of the organisms was inoculated into a 250ml of Erlenmeyer flask containing 50ml fermentation medium (glucose 10.0; yeast extract 2.0; KH_2PO_4, 1.0; $MgSO_47H_2O$, 0.5; $CaCl_2.2H_2O$, 0.2; peptone 10.0; $(NH_4)_2SO_4$, 1.0g; H_2O 1L, pH 7.0). The flask was incubated for 72h at 35^0C on a rotary shaker (150rpm). All experiments were performed in duplicate.

Protease activity assay

Protease activity was assayed using the casein-digestion method of Hameed *et al*. (1996). To 1ml of 1% casein solution pH 8.5, 1ml of crude enzyme solutions was added and incubated for 30min at 40^0C. The reaction was stopped by adding 3ml of 5% trichloroacetic acid (TCA). After, 30min the supernatant was separated by centrifugation at 10,000 xg for 30min and absorbance of amino acids and peptides released in a spectrophotometer (Cecil CE 1020). All protease activity assays were made in duplicates. Blanks were prepared in which 3ml of trichloroacetic acid was added to the sample before incubation. One unit of protease activity was defined as the amount of enzyme that will release 10µg of tyrosine under the specified assay conditions (pH 8.5, 40^0C and 30min).

RESULTS

The number of *Bacillus* species isolated from the soil in Awka is presented in table 1. The number isolated ranged from 5-15, with the soil at Amaenyi having the highest of 15 and the soil at Ifite Awka having the lowest of 5.

The *Bacillus* species isolated (Table 2) included *B. licheniformis, B. subtilis, B. megaterium, B. stearothermophilus, B. macerans, B. coagulans, B. pumilus* and atypical strains. *B. licheniformis* was the most common (24.1%) while atypical strains were the least common (5.56%).The percentage occurrence for *B. megaterium, B. macerans* and *B. coagulans* was 9.26%.

The result of the screening for protease production by *Bacillus* species is shown in table 3. All the *Bacillus* species screened exhibited ability to produce protease. The highest zone of clearance was observed in *Bacillus subtilis* SE2 (12.3mm), *B. subtilis* SU8 (10.8mm) and *B. licheniformis* SE9 (10.2mm). *Bacillus macerans* produced the lowest zone of clearance of 4.3mm. The 3 highest protease producers were selected for further study.

The results of submerged production of protease by Bacillus species is presented in Table 4. The highest protease production of 102 and 133 U/ml by *Bacillus subtilis* SE2 and *B. subtilis* SU8 was recorded after 48h, while *B. licheniformis* SE9 accumulated the highest protease yield of 148 U/ml after 72h of incubation.

DISCUSSION

In the study *Bacillus* species were isolated from all the soil samples. This confirms that the organism is ubiquitous in nature and it is thought to contribute substantially to nutrient cycling due to the diversity of enzymes produced by members of the genus. Watanabe and Hayano (1993), identified *B. subtilis, B licheniformis, B.cereus* and *B. megaterium* in soil isolations. In another study, Waksman (1961), identified 29 isolates of *B. megaterium* and 24 isolates of *B. subtilis* out of 306 soil samples. Of the 54 *Bacillus* species examined, 3 had characteristics that did not confirm with the known species. The existence of these atypical strains agrees with the work of Gordon *et al*. (1973), who could not assign 77(12.60%) of the 607 strains of *Bacillus* to any known species. Again, Westhoff and Dougherty (1981) were able to isolate some atypical strains in ultra-high temperature processed milk.

The screening for protease production in the study showed that *Bacillus subtilis* SE2 produced the highest protease activity with a zone of clearance of 12.3mm, followed by *B. subtilis* SU8 with a zone of clearance of 10.8mm. This is in contrast to the report of Olajuyigbe and Ajele (2005), who observed that *B. licheniformis* IKBL–17 produced the highest activity followed by *B. subtilis* IKBS-10. However, it was noticed that the protease producing ability of *Bacillus* species could vary quite considerably. This was shown by the relatively wide range (4.3–12.3mm) of the means of the zones of clearing observed around the colonies of the *Bacillus* species. Several species of *Bacillus* have been isolated that produce neutral or alkaline protease (Puvanakrishnan and Dhar, 1986).

Quantitative assessment of proteolytic activity during submerged fermentation, showed that *B. subtilis* SE 2 and SU 8 accumulated highest protease level after 48h, while *B. subtilis* SE 9 produce the highest quantity after 72h.The maximum production of protease by *B. subtilis* SE 2 and SU 8 after 48h is in agreement with the work of Hameed *et al*., (1996) who noted that the *B. subtilis* used was able to produce the highest quantity of the protease enzyme

Table 1: Number of *Bacillus* species isolated from soils at different locations in Awka town

Location	Soil colour	Texture	Number of *Bacillus* species
Amaenyi	Black brown	Coarse	15
Government house	Brown	Sandy	10
Amawbia	Brown	Fine	8
Agu Awka	Black brown	Coarse	6
Ifite Awka	Slight brown	Coarse	5
Eke Awka	Brown	Sandy	10
Total			54

Table 2: Percentage occurrence of *Bacillus* species isolated from the soil

Bacillus species	Number(%) occurrence
B. licheniformis	13(24.1)
B. subtilis	11(20.4)
B. megaterium	5(9.26)
B. stearothermophilus	8(14.8)
B. macerans	5(9.26)
B. coagulans	5(9.26)
B. pumilus	4(7.41)
Atypical strains	3(5.56)
Total	54(100)

Table 3: Screening for protease production by *Bacillus* species

Bacillus species	Average zone of clearance(mm)
Bacillus licheniformis SE9	10.2
B. licheniformis SU7	7.5
B. licheniformis SAK3	9.3
Bacillus subtilis SE2	12.3
B. subtilis SU8	10.8
B. subtilis SE4	6.5
Bacillus megaterium SE3	8.5
B. megaterium SA2	9.0
B. megaterium SU3	6.8
Bacillus stearothermophilus SUG 5	9.3
B. stearothermophilus SAK 2	8.2
B. stearothermophilus SA9	5.8
Bacillus pumilus SU9	5.3
B. pumilus SIK 9	9.0
B. pumilus SE 11	7.0
Bacillus macerans SA 1	4.3
B. macerans SAK 5	7.5
B. macerans SE 14	8.0
Bacillus coagulans SUG 6	5.0
B. coagulans SU 10	7.0
B. coagulans SIK 2	6.8
Atypical strain SAK 7	7.4
Atypical strain SU1	8.1

Table 4: Submerged production of protease by Bacillus species

Bacillus species		Protease activity (Units/ml)			
		24h	48h	72h	96h
B. subtilis	SE 2	64	102	91	73
B. subtilis	SU 8	87	133	115	98
B. licheniformis	SE 9	77	108	148	109

after 48h of fermentation. Again, Sookkheo *et al*. (2000) and Fujio and Kume (1991) were able to produce the highest quantity of protease with *B. stearothermophilus* after 48h of fermentation.

In conclusion, the result of the study shows that protease producing *Bacillus* species abound in the soil.

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