Protease Production by Submerged Fermentation in Shake Flasks Using *Bacillus subtilis* Isolated From the Soil

ABSTRACT

Proteases are one of the largest and most important industrial enzymes which account for about 60% of total enzyme market. Protease production by submerged fermentation in shake flasks using *Bacillus subtilis* isolated from the soil was studied. Soil samples were collected from different refuse dump sites located at Uli, Ihiala LGA in Anambra State. One gram of each soil sample was transferred into a test tube containing 9 ml sterile diluent. A ten fold serial dilution was conducted and 0.1 ml of 10^{-4} dilution was inoculated into skimmed milk agar plates and incubated at 30 ^{o}C for 72 h. A clear zone around the colonies gave an indication of protease-producing bacterial isolates. The selected protease producers were subsequently used for shake flask fermentation in 50 ml sterile medium. Optimization study was conducted to determine the effect of carbon sources , nitrogen sources, trace elements, agitation rates and pH. Twenty one bacteria isolates were found to be active protease producers and isolates RS-5 and OS-9 had the highest zone of clearance of 13.5 mm and 12.1 mm respectively. The result of submerged production of protease by the bacteria isolates revealed that the isolates RS-5 and OS-9 accumulated maximum protease yield of 3.23 and 2.71 U/ml respectively. The isolates were Gram positive and endospore formers, and were identified as *Bacillus subtilis*. The addition of Starch and maltose stimulated optimum protease production of 3.47 U/ml and 2.77 U/ml by *Bacillus subtilis* RS-5 and OS-9 respectively. Beef extract enhanced maximum enzyme yield of 3.35 and 2.9 U/ml for *Bacillus subtilis* RS-5 and OS-9 respectively. Maximum protease yield of 3.28 U/ml for *Bacillus subtilis* RS-5 and 2.85 U/ml for *Bacillus subtilis* OS-9 was obtained by the supplementation of 0.4 g/l of $FeS0_4$ respectively. The maximum protease yield was observed at agitation rate of 200 rpm for *Bacillus subtilis* RS-5 and 170 rpm for *Bacillus subtilis* OS-9. At pH8, protease accumulation was highest for *Bacillus subtilis* RS-5 and OS-9. The study revealed that the soil harbour some protease-producing bacteria strains. Optimization studies conducted on the bacteria isolates showed that protease production can be greatly enhanced.

Keywords**:** Soil, bacteria, protease, trace elements, submerged production

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1. INTRODUCTION

Proteases are degradative enzymes, which catalyzes the total hydrolysis of protein [1] and are classified according to their structure or the properties of the active site [2]. There are several kinds of proteases such as serine, metallo, carboxyl, acidic, neutral and alkaline proteases [2].

Protease constitutes one of the most important group of industrial enzymes accounting for about 60% of the total worldwide enzyme sales [3, 4, 5] . Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases. And among bacteria, *Bacillus* species are specific producers of extracellular proteases. Microbial proteases are preferred to proteases from plant and animal sources for various reasons, which include the development of eco-friendly technology. Proteases are one of the most important industrial enzymes and are used in a variety of industrial applications, such as laundry, detergents, pharmaceutical industry, leather industry in dehairing and bating of hides, manufacture of the protein hydrolysis,

food industry like meat tenderizing, cheese flavor development, treatment of flour in the manufacture of baked goods, improvement of dough texture, flavor and colour in cookies [6, 7, 8], silver recovery from X–ray film and even in waste processing industry [9, 10]. Protease is not produced locally and the cost of procurement is very high. The local production of the enzyme will save the huge amount of foreign exchange spent annually for its importation.

This study was undertaken to isolate protease producing bacteria and optimize the culture conditions for optimal protease yield.

2. MATERIALS AND METHODS

Soil samples were randomly collected from Ten different refuse dump sites located in Anambra State University Uli and taken to the laboratory for analysis.

2.1 Isolation and screening of bacteria from soil

One gram of each soil sample was transferred into a test tube containing 9 ml normal saline. A ten fold serial dilution was conducted and 0.1 ml of 10^{-4} dilution was inoculated into skimmed milk agar plates and incubated at 30 ^{o}C for 72 h. A clear zone around the colonies gave an indication of protease-producing bacterial isolates. The protease producers were subcultured severally into nutrient agar plates to obtain pure cultures. The pure isolates were inoculated on skimmed milk agar plates and incubated 30 ^{o}C for 72 h. The diameters of the clear zones around the colonies were measured using a ruler. A total of 88 isolates were screened and only 21 were found to be protease producers. These active protease producers were then used for submerged fermentation.

2.2 Protease production in shake flask fermentation

A loopful of the selected isolate was taken from agar slant and inoculated into 20 ml of sterile nutrient broth and incubated at 30 ^{0}C for 24 h. This served as the seed inoculum for the fermentation. A 250ml Erlenmeyer flask containing 50ml of fermentation medium g/l: glucose, 5; peptone, 7.5; casein, 1; $MgS04.7H_2O$, 5; KH_2PO_4, 5; $FeSO_4$ $7H_2O$, 0.1; pH 7.2 was sterilized and inoculated with 2 ml of a 24 h seed inoculum of each selected isolate. The flask was incubated at 30 ^{0}C on a rotary shaker (160 rpm) for 72 h. After incubation the cell broth was centrifuged at 5000 rpm for 15 min. The cell free supernatant was used for protease determination.

2.3. Identification of the bacterial isolate

The bacterial isolates that showing the highest L-glutaminase production were identified on the basis of morphology and biochemical characteristics, including endospore staining.

2.4 Determination of protease activity

Protease activity was measured using the casein digestion method of [11]. One unit of protease activity was defined as the amount of enzyme that will release 10µg of tyrosine under the specified conditions (pH 8.5, 40^{0}C and 30min).

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2.5 Determination of bacterial growth

The bacteria growth was determined turbidimetrically from the culture broth in a spectrophotometer at 660nm.

2.6 Optimization of medium components on protease production

2.7 Effect of carbon sources

The effect of various carbon sources(0.5%w/v) which included maltose, glucose, starch, lactose and mannitol was studied by growing the organism in fermentation media containing each of them. The flasks were incubated at 30^0C on a rotary shaker (160rpm) for 72h. After incubation the cell broth was taken for determination of bacteria growth and protease production.

2.8 Effect of nitrogen sources

The effect of various nitrogen sources(1%w/v) which included gelatine, soybean, tryptone, peptone, beef extract, casein and yeast extract was studied by growing the organism in fermentation media containing each of them. The flasks were incubated at 30^0C on a rotary shaker (160rpm) for 72h. After incubation the cell broth was taken for determination of bacteria growth and protease production.

2.9 Effect of trace elements

Effect of different concentrations of trace elements (3 – 10g/l) of $MgSO_4$ and KH_2PO_4 and (0.1 – 0.4 g/l) of $FeSO_4$ on protease production was determined by growing the organism in fermentation media containing each of them. The flasks were incubated at 30^0C on a rotary shaker (160rpm) for 72h. After incubation the cell broth was taken for determination of bacteria growth and protease production.

2.10 Effect of agitation rates

Effect of agitation rates on growth and protease production was studied by growing the organism in fermentation medium at different agitation speed (80-200rpm) at 30^0C for 72h. After incubation the cell broth was taken for determination of bacteria growth and protease production

2. 11 Effect of pH

Effect of pH values on growth and protease production by *Bacillus* species was studied by growing the organism at different pH values (6-9) of the fermentation media. The flasks were incubated at 30^0C on a rotary shaker (160rpm) for 72h. After incubation the cell broth was taken for determination of bacteria growth and protease production

Table 1: Qualitative Screening for protease-producing bacteria using solid agar

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Bacteria isolate code	Gram reaction	Spore test	Average zone of clearance (mm)
RS-2	-cocci	-	9.0
IS-10	-rods	+	7.2
US-9	+cocci	-	3.0
US-6	+rods	+	9.3
RS-1	-cocci	-	3.8
OS-13	+cocci	-	3.5
RS-4	+rods	+	6.4
US-2	+rods	+	11.0
RS-5	+rods	+	13.5
RS-11	+cocci	-	6.3
IS-3	-cocci	-	7.7
IS-6	+rods	+	9.6
IS-2	-rods	-	5.3
OS-7	+cocci	-	4.1
OS-9	+rods	+	12.1
IS-5	-cocci	-	4.0
US-3	+rods	+	9.0
RS-7	-rods	-	5.3
RS-8	-rods	-	8.2
OS-2	-cocci	-	4.0
US-4	+rods	+	6.2

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Table 2: Shake flask production of protease by bacteria isolated from soil

Bacteria isolate code	Protease activity(U/ml)

RS-2	1.35
IS-10	0.85
US-9	0.27
US-6	1.74
RS-1	0.67
OS-13	0.44
RS-4	1.31
US-2	2.45
RS-5	3.23
RS-11	0.53
IS-3	0.77
IS-6	2.18
IS-2	0.41
OS-7	0.55
OS-9	2.71
IS-5	0.93
US-3	1.84
RS-7	1.10
RS-8	1.50
OS-2	0.37
US-4	1.11

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Table 3: Effect of carbon sources on growth and protease production by *Bacillus subtilis* RS-5 and OS-9

Carbon source(0.5% w/v)	*Bacillus subtilis* RS-5		*Bacillus subtilis* OS-9	
	Protease activity (U/ml)	Bacterial growth (OD660nm)	Protease activity (U/ml)	Bacterial growth (OD660nm)
Maltose	3.12	1.93	2.77	1.47
Glucose	3.04	1.88	2.56	1.40
Starch	3.47	1.97	2.60	1.32
Lactose	2.91	1.76	2.44	1.47
Mannitol	2.62	1.60	2.25	1.30

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Table 4: Effect of nitrogen sources on growth and protease production by *Bacillus subtilis* RS-5 and OS-9

Nitrogen Source (1% w/v)	*Bacillus subtilis* RS-5		*Bacillus subtilis* OS-9	
	Protease activity (U/ml)	Bacterial growth (OD660nm)	Protease activity (U/ml)	Bacterial growth (OD660nm)
Gelatine	2.76	1.75	2.44	1.24
Soyabean meal	2.21	1.58	1.93	1.05
Tryptone	3.03	1.84	2.58	1.36
Peptone	3.23	1.89	2.71	1.45
Beef extract	3.35	2.03	2.90	1.57
Casein	3.16	1.85	2.76	1.41
Yeast extract	3.28	1.80	2.64	1.35

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Table 5: Effect of trace elements on growth and protease production by *Bacillus subtilis* RS-5 and OS-9

Trace element	Concentration (g/l)	*Bacillus subtilis* RS5		*Bacillus subtilis* OS9	
		Protease activity (U/ml)	Bacteria growth (OD66onm	Protease activity (U/ml)	Bacteria growth(OD660nm)
MgS04	3.0	2.58	1.61	2.36	1.41
	5.0	2.86	1.67	2.49	1.45
	7.0	2.97	1.75	2.72	1.52
	10.0	3.25	1.86	1.85	1.57
KH_2PO4	3.0	2.70	1.57	2.03	1.38
	5.0	2.93	1.66	2.19	1.43
	7.0	2.26	1.70	2.28	1.46
	10.0	2.13	1.78	1.72	1.37
FeS04	0.1	2.43	1.67	2.51	1.33
	0.2	2.60	1.73	2.68	1.41
	0.3	2.86	1.84	2.79	1.46
	0.4	3.28	1.72	2.85	1.51

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Table 6: Effect of agitation rate on growth and protease production by *Bacillus subtilis* RS-5 and OS-9

Agitation	*Bacillus subtilis* RS-5	*Bacillus subtilis* OS-9

Rate(rpm)	Protease activity (U/ml)	Bacteria growth (OD660nm)	Protease activity (U/ml)	Bacteria growth (OD660nm)
80	1.51	1.60	0.97	1.31
100	1.74	1.63	1.20	1.34
120	2.27	1.65	1.50	1.42
150	2.96	1.70	2.18	1.49
170	3.30	1.77	2.86	1.54
200	3.45	1.82	2.85	1.63

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Table 7: Effect of pH on growth and protease production by *Bacillus subtilis* RS-5 and OS-9

Ph	*Bacillus subtilis* RS-5		*Bacillus subtilis* OS-9	
	Protease	Bacterial growth	Protease	Bacterial growth

	activity (U/ml)	(OD660nm)	activity (U/ml)	(OD660nm)
6.0	1.97	1.750	1.88	1.24
6.5	2.81	1.78	2.44	1.31
7.0	3.03	1.84	2.58	1.36
7.5	3.23	1.89	2.71	1.45
8.0	3.40	2.03	2.90	1.46
8.5	3.16	1.85	2.76	1.41
9.0	2.90	1.80	2.14	1.27

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3. RESULTS

In all the bacteria isolated and screened, nine of the bacteria isolates were Gram positive rods, 3 were Gram negative rods,4 were Gram positive cocci and 5 Gram negative cocci. The spore formers isolated were 9 in number. The screening for protease production shows that isolate RS5 and OS9 had the highest zone of clearance of 13.5mm and 12.1 mm respectively (Table 1). The result of shake flask production of protease by bacteria as shown in Table 2 revealed that the isolates RS5 and OS9 accumulated maximum protease yield of 3.23 and 2.71 U/ml respectively.

Two bacterial isolates showing the highest L-glutaminase production were identified on the basis of morphology and biochemical characteristics, including endospore staining. They were identified as *Bacillus subtilis* RS5 and OS9.

Table 3 shows the effect of carbon sources on growth and protease production by *Bacillus* species. Starch and maltose stimulated maximum growth and protease production of 3.47 U/ml and 2.77 U/ml by *Bacillus* species RS5 and OS9 respectively, while mannitol encouraged the least accumulation(2.62 U/ml and 2.25 U/ml) in both species

The effect of nitrogen sources on growth and protease production by *Bacillus* species is shown in Table 4. Beef extract enhanced maximum growth and enzyme yield of 3.35 and 2.9 U/ml for *Bacillus* species RS-5 and OS-9 respectively, while soyabean meal stimulated the least production of 2.21 and 1.93U/ml in both isolates.

The effect of medium trace elements on growth and protease production (table 4) showed that maximum protease yield for isolates RS5 and OS9 was obtained by MgSO4 at a concentration of 10 and 7 g/l respectively. KH_2PO_4 at a concentration of 5 and 7 g/l stimulated optimum protease production in RS5 and OS9 respectively, while $FeSO_4$ at a concentration of 0.4 g/l stimulated protease production in both isolates.

The result of the effect of agitation on growth and protease production is shown in table 5. Maximum protease yield was obtained at 200rpm (3.45 U/ml) for isolate RS5, while maximum yield was obtained by isolate OS9(2.86 U/ml) at 170 rpm.Maximum bacteria growth for both isolates was observed at 200 rpm.

Table 3 shows the result of effect of pH on growth and protease production is shown in Table 4. The highest protease yield for isolates RS5 (3.40 U/ml) and OS9 (2.90 U/ml) was achieved at a pH 8.0, there was decrease in protease yield at pH 8.5 and 9 respectively. The bacteria growth for both isolates decreased at pH 8.0.

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4. DISCUSSION

A total of 21 bacterial organisms isolated from the soil were found to be protease producers. The occurrence of protease producing organisms from the soil agrees with the report of [1], who isolated protease producers from soil. [12] , isolated proteolytic bacteria from soil samples of Ikogosi warm spring. .

In the study starch stimulated maximum protease production in *Bacillus* species. This is corroborated by the report of [13] , who showed that starch was the best carbon source for growth and protease production by *Bacillus* species. Similarly, [14] also reported that starch caused high level of expression in *Bacillus* species. [15], reported that among the ten carbon sources studied, starch, sucrose and lactose proved appreciably good for the protease

production. In constrast, [16], reported that wheat bran supported the maximum production of protease by *Bacillus* species.

The results obtained revealed that beef extract supported enhanced protease production. This is similar to the findings of [14], who observed that beef extract enhanced protease production by *Bacillus cereus* strain 146. Also, [16], reported that beef extract was the best nitrogen source for protease production by *Bacillus* species. In contrast, [17] , found skim milk to have significant effect on the production of protease by *Bacillus cereus* strain CA15.

Different concentration of trace elements ($MgSO_4$, KH_2PO_4, $FeSO_4$) used in the study encouraged growth and protease yield. [1] reported that combination of Ca^{2+} and Mg^{2+} in medium stimulated the highest protease production by *Bacillus* sp. N-40 isolated from the soil, but noted that both ions were not effective alone. Trace elements play a vital role in fermentation as they are required to activate enzymes [18], Fe^{2+} and Mn^{2+} seem to be the most important of the trace elements as they play a role in the excretion of primary metabolites.

In the study *Bacillus* species RS5 and *B.* speciesOS9 were observed to produce maximum protease at agitation rate of 200 and 170rpm respectively. [2] and [19] reported maximum protease production at 200rpm using *Bacillus licheniformis*NCIM-2042 and *B. subtilis* strain Rand respectively. For *Bacillus* species OS9, It was observed that at 200 rpm protease production reduced this could be as a result of excessive agitation which may lead to cell lysis and denaturation of enzymes. At the speed of 170 and 200rpm, it is opined that aeration of the culture medium was increased which could lead to sufficient supply of dissolved oxygen in the media [20]. Nutrient uptake by bacteria also would be increased [4] resulting in increased protease production. [14], pointed out that mixing is especially important because oxygen is a very low solubility nutrient. Agitation intensity provide homogeneity and influences the oxygen transfer rate in many bacterial fermentations thereby influencing growth and product formation.

The result revealed that optimum protease yield was achieved at pH 8.0 by the isolates. This corroborate the report of [21] who considered the pH of 8.0 as the best pH for protease production by *Bacillus subtilis.* Also [12], observed maximum protease production at pH 8.0. In contradiction, [16] observed maximum protease production at pH 9 for *Bacillus* species K-30 using rice bran. The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. [22], reports maximum enzyme production was observed in the culture medium of pH 9.0. However, a relatively high enzyme yield was achieved between pH 7 and pH 10. A similar result (pH 9.0) and closely related one (pH 10) were obtained from proteases produced from a *Bacillus* species and *B. halodurans* [23, 16]. Generally, pH of a culture medium affects both the morphological and physiological characteristics of an organism. [24] and [25] observed maximum lipase at a pH 8. Microorganisms vary in their oxygen requirements. In particular, oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities [3].

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