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Production of protein rich organic fertilizer from fish scale by a mutant *Aspergillus niger* AB_{100}– A media optimization study

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Fish scale, the chief waste material of fish processing industries, was enzymatically hydrolyzed by protease of *Aspergillus niger* AB_{100} to produce soluble protein for use as organic fertilizer. Optimum inorganic nutrients were found to be: $MgSO_4$, 0.75; K_2HPO_4, 0.2; and KCl, 0.075%. *A. niger* AB_{100} required $FeSO_4.7H_2O$ (5 μg/ml) and $ZnSO_4.7H_2O$ (15 μg/ml) for higher amount of protease production and yield. The optimum values for the tested cost-effective nutrients for the maximum protease production were: Defatted soybean-meal, 0.2; Peptone, 0.3; and Corn-steep-liquor, 0.2%. Biomass growth was not related proportionately in every case with that of enzyme production and protein solubilisation but protease production shows a positive correlation with hydrolysis of scale to produce soluble protein.

Keywords: Protein rich organic fertilizer, Fish scale, Protease, *Aspergillus niger* AB_{100}
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Introduction

With rapid progress of civilization, demand of low cost protein foods is gradually increasing. Waste products from food industries having proteinacious materials have invited researchers to explore new non-conventional resources of protein. Attempts have been made to use waste material of food processing industries like agriculture[1], poultry, meat and fish industries[2-4] for recovery of protein. Annually over 100 million tons of fish are harvested world wide, and about half of the total catch is discarded as processing waste[5]. From 1987 to 1996, increase in aquaculture production[6] is: World, 148; and, India, 126%. In case of *Labeo rohita* (23.9% of the total production in India)[7] there was 131 percent increase in production. In this species, the whole body (with the exception of the head and fins) is protected by a number of regularly arranged thin flexible cycloid scales, overlapping one another and comprising of about 6 % (dry weight) of the wet weight of fish[8]. Thus, for each kg of fish consumed, 60 g of scales, which contains protein mainly in the form of collagen and icthylpeden[9] with little amount of Ca, Mg and P along with traces of Na and S[10], are wasted. Several extraction processes and enzymatic hydrolysis are reported for recovery of protein from fish scale[11-13]. But reports on microbial recovery of protein from fish scale is lacking.

Microbial proteases can hydrolyse proteins from plants, fish, or animals to produce hydrolysates of well-defined peptide profile, such as protease of *Bacillus amyloliquefaciens* produced methionine-rich protein hydrolysate from chickpea protein[14], *Bacillus subtilis* alkaline protease produce protein concentrate from waste feathers from poultry slaughter house[15], keratinolytic alkaline proteases were used in feed technology for production of peptides from waste keratinous material[16,17], degradation of wool by *Aspergillus niger* and *Trichophyton simii*[18] and production of soluble protein from waste leather by protease of mutant *A. niger*[19].

This study presents the effect of various cost-effective nutrients, requirements of trace metals and essential inorganic nutrients on a mutant strain of *A. niger* AB_{100} for the higher amount of protease yield and production of soluble protein from fish scale.

Materials and Methods

Microorganism

The parent strain of *A. niger* was isolated from North-Bengal soil and explored to ethylene-imine and X-rays (35 KV & 10 Ma) for development of a mutant strain[20] of *A. niger* $AB_{100.}$ This mutant strain was subcultured for 15 generations on Czapek dox agar slants[21]. During experiment, cultures were transferred to slants of malt and

yeast extract agar medium[22,23] and incubated at 28°C for 7 d for sufficient sporulation.

Spore suspension was prepared by harvesting the spores by washing the slants with 10 ml sterile triple distilled water and filtering the resulting spore suspension through several layer of cotton[24]. The density was adjusted to 1.4 x 10^7 spores/ml of spore suspension. This suspension (5 ml) was used as inoculum during surface culture fermentation.

Processing of Fish Scale

In the present study, fish scale of *L.rohita* were collected from market and washed with deionised water, dried in mechanical drier for 12 h at 100°C. Then, after adding required quantity of distilled water, scales (1g scale/ml of water) were steamed for 30 min prior to fermentation. This is necessary because denaturation of the collagen structure helps to moisture uptake and improves the diffuusibility of enzyme in scale substrate to increase its susceptibility to hydrolysis[20]. After steaming, scales were dried in mechanical drier and then crushed to almost powdered form. Crushed scale (2.5 g) was used in fermentation experiment.

Medium and Cultural Conditions

Surface culture fermentation was carried out using 150 ml conical flask, which contained 30 ml fermentation medium (FM) consisting of: glucose, 0.05; urea, 0.025; $MgSO_4.7H_2O$, 0.01; and KH_2PO_4, 0.01 g/ml; processed fish scale, 2.5 g; and pH, 4.0. Inoculated medium were incubated at 28 ºC (± 0.5 ºC in BOD incubator) for 14 d. Sterile solution of $MgSO_4.7H_2O$, K_2HPO_4, KH_2PO_4, NaCl, KCl and $CaCl_2$ added individually to the sterile medium keeping all the factors constant except one, effects of which was under investigation. Sterile solutions of trace metals were added separately with glucose, urea, $MgSO_4.7H_2O$ and K_2HPO_4 solutions. Chemicals obtained from Qualigen Excelar were of analytical grade and were further purified from trace elements by the method of chloroform extraction[24,25]. In this method, the required amount of aqueous solution of each was mixed well with 0.1 g of 8-hydroxyquinone and chloroform in a separating funnel, first at pH 5.2, then at pH 7.2. The chloroform-dissolved impurities were extracted out. The process was repeated at two different pH. The solutions were made chloroform free by heating in a water bath and sterilized separately. Complex nutrients were added as sterile solution based on their solid content.

Determination of Nitrogen Content of Scale

Nitrogen content of fish scale was measured before and after fermentation by modified micro-kjeldahl digestion method[26]. The percentage of solubilised nitrogen was calculated comparing the nitrogen value of degraded and non-degraded scale. Protein content of scale was obtained by multiplying nitrogen content with the factor 6.25.

Enzyme Assay

After fermentation, mycelium was separated from FM by filtration through Whatman no. 1 filter paper and 0.5 ml of 1:10 diluted clear filtrate was taken to determine protease activity. The enzyme activity was determined spectrophotometrically using casein as a substrate[27]. One unit of enzyme activity was defined as the amount of enzyme liberating one µ mole of tyrosine/ml/min under the defined conditions.

Estimation of Protein and Dry Mycelial Mass

Protein was estimated by the method of Lowry[28] using Folin-ciacalteau reagent and bovine serum albumin as standard. The mycelia, separated from the fermentation liquor by filtration, washed with distilled water and filtered once again through a Buchner funnel, were dried in an oven for 24 h at 80 ºC. It was weighted to the nearest gram to obtain the quality of dry mass[29,30].

Statistical Analysis

Statistical analyses of all data were performed according to student's t distribution[31]. The level of significance for two-tail test was determined from the table with critical values of t. The number of samples was 6.0 (n=6).

Table 1 — Effect of $MgSO_4.7H_2O$ on protease production during solubilisation of protein from fish scale by *A niger*AB_{100}

Conc Of $MgSO_4.7H_2O$	Solubility of fish scale protein*	Dry mycelial weight	Soluble protein in FM	Protease activity[a]
%	%	g/100 ml	g/l	unit/ml/min
0.50	36.0 ± 0.603	3.00	5.10	1014.00
0.75	40.7 ± 0.266	3.20	9.50	1280.00
1.00	39.0 ± 0.569	3.20	7.90	1186.00
1.25	38.0 ± 0.443	3.25	7.00	1140.00
1.50	36.1 ± 0.326	3.30	5.20	1020.00
2.00	33.9 ± 0.418	3.30	3.30	910.00

* = Values are expressed as mean ± standard error, n=6.
[a] = protease activity was significantly different from each other ($p<0.001$)

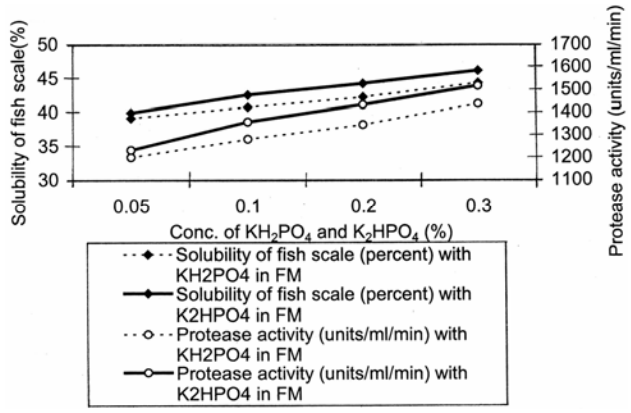

Fig. 1— Effect of KH_2PO_4 and K_2HPO_4 on protease production and protein solubilisation from fish scale by *A. niger* AB_{100}

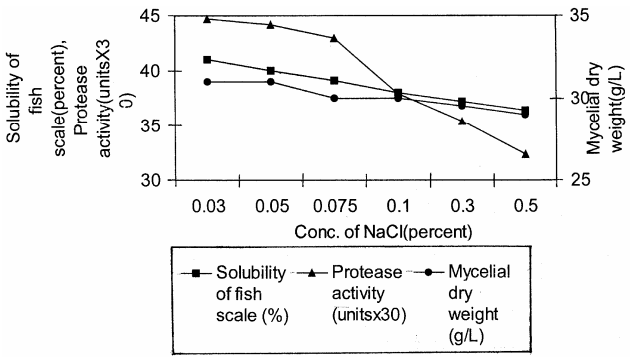

Fig. 2(a) —Effect of NaCl on protease production by *A. niger* AB_{100} during solubilisation of protein from fish scale

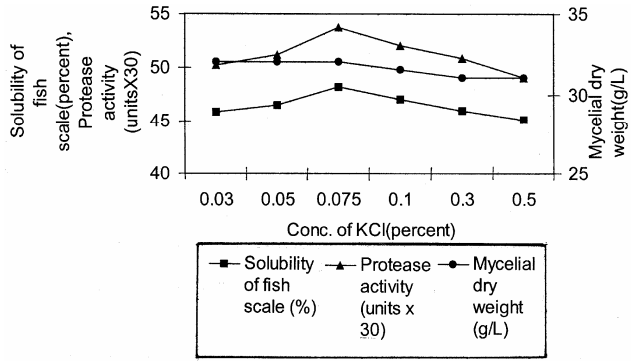

Fig. 2(b) — Effect of KCl on protease production by *A. niger* AB_{100} during solubilisation of protein from fish scale

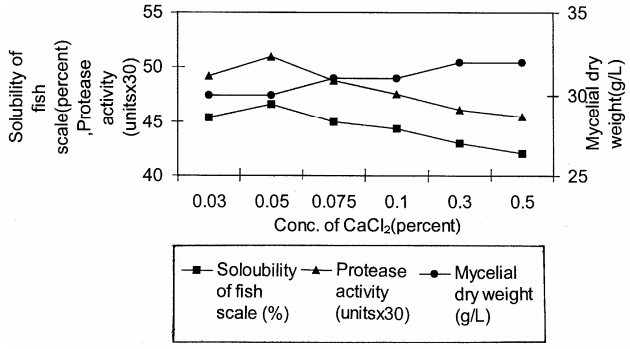

Fig. 2(c) —Effect of $CaCl_2$ on protease production by *A. niger* AB_{100} during solubilisation of protein from fish scale

Results and Discussion

Effects of $MgSO_4.7H_2O$

Growth of *A. niger* was proportional to Mg content of the medium, which was present as sulphate. Different concentrations of $MgSO_4.7H_2O$ (0.5, 0.75, 1.0, 1.25 & 1.5 %) were tested and 0.75 per cent (Table 1) gave highest protease production and scale solubilisation, which was significantly ($p<0.001$) higher than control. Mg^{2+} functions by influencing fungal enzyme system and protease is one of them[32,33].

Effects of KH_2PO_4 and K_2HPO_4

Potassium phosphate, used as a source of phosphate,[34,35] was responsible for buffering the medium[36]. Two different inorganic phosphate sources were added to FM in different concentrations (0.1, 0.2, 0.3, 0.4, 0.5 & 0.6

Table 2— Combinational effect of Fe^{3+} and Zn^{2+} on protease production and fish scale solubilisation by *A niger* AB_{100}.

Conc. of Zn $SO_4.7H_2O$ µg/ml	Conc. of $FeSO_4.7H_2O$ µg/ml	Solubility of fish scale protein* %	Soluble protein in FM g/l	Protease activity[a] units/ml/min
15.00	1.00	52.5 ± 0.871	21.10	1846.00
15.00	5.00	54.8 ± 0.833	23.30	2020.00
15.00	10.0	52.2 ± 0.884	20.80	1828.00
15.00	15.0	50.6 ± 0.804	19.40	1748.00
Conc. of $FeSO_4.7H_2O$	Conc. Of $ZnSO_4.7H_2O$			
15.00	1.0	48.0 ± 0.761	16.70	1600.00
15.00	5.0	49.6 ± 0.630	18.30	1690.00
15.00	10.0	51.4 ± 0.577	20.20	1780.00
15.00	15.0	50.0 ± 0.720	18.70	1720.00

* = Values are expressed as mean ± standard error, n=6.
a = protease activity was significantly different from each other ($p<0.001$)

%), between them, 0.3 per cent K_2HPO_4 (equivalent to 0.00534% P) exhibited optimum result (Fig. 1), however, 0.2 per cent P has been reported to be optimum for protease production[13,36] . Amount in excess of 0.3 per cent showed an inhibition on cell growth and repression in protease production.

Effects of NaCl, KCl and $CaCl_2$

Different concentrations of NaCl, KCl and $CaCl_2$ (0.03, 0.05, 0.075, 0.1, 0.3 & 0.5 %) were added to FM. There was a strong inhibitory effect of NaCl on

protease production, protein solubilisation and cell growth (Fig. 2a). KCl (0.075%) has a stimulatory effect on total solubilisation process (Fig. 2b) and significantly higher ($p<0.001$) than control. $CaCl_2$ (0.05%) has slight positive effect on enzyme production, however, higher concentration imparts a negative effect (Fig. 2c).

Results of this study with NaCl were in agreement with previous observations[32,37], where sodium salts like NaCl, Na_2SO_4 and Na_2S depressed proteolytic activity of microorganisms. Growth impairment of *A. niger* AB_{100} with KCl (< 0.075 %) in present study, may be due to oxalic acid accumulation as well as non-acid tolerance nature of *A niger*[32,33,35]. $Ca2^{+}$(0.5-2.0 ppm) has been recognized as inhibitory to fungal growth[32]. Ca^{2+} resulted in partial inhibition in case of alkaline protease of *P. aeruginosa* MN1[38] and 0.1% $CaCl_2$ was optimum for waste collagen degradation by *A.niger*[33].

Effects of Trace Metals

Eight essential trace metals (Fe^{3+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Vo^{3+}, Mo^{+}, Cu^{2+} & Ni^{2+}) of fungi were tested at a conc. of 5, 10, 15 and 20 µg/ml. $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$ (15µg/ml) have significant stimulatory effects on enzyme production and protein yield. To explore the combinational effect of Fe^{3+} and Zn^{2+}, another experiment was performed keeping either one constant and at a time varying the other. Keeping fixed conc. of $ZnSO_4.7H_2O$ (15µg/ml) and varying concentration of $FeSO_4.7H_2O$ (5µg/ml in this case) exerts more stimulatory effect on protease production and protein solubilisation (Table 2). This result differs significantly ($p<0.001$) over previous experiment where Fe^{3+} and Zn^{2+} affect individually and there was increase of protease activity (84 units) and in protein solubilisation (2.1 %).

Divalent metal ions (calcium, copper, cobalt, boron, iron, manganese, molybdenum) are required in fermentation medium for optimum production of protease[13,36]. Iron, being an integral part of the fungal protoplasm, is associated with various enzyme systems[32] . Spore pigment of black *A. niger* aspergillin contains 0.26 percent iron[33]. These facts may be the cause of stimulatory effect of Fe^{3+} of this study, which was supported by previous report[33]. Positive effects of Zn^{2+} (15µg/ml) in present study may be due to Zn^{2+} acting as an activator or constituent of protease[32]. Higher conc. of Zn^{2+} (>15 g/ml) has inhibitory effect on *A. niger* due to toxic effects of Zn^{2+} like mutagenic change in fungal organism[33]. $ZnSO_4$ at a conc. of 0.125 mg/100ml resulted an increase in protease activity by *R. oryzae*[39,40].

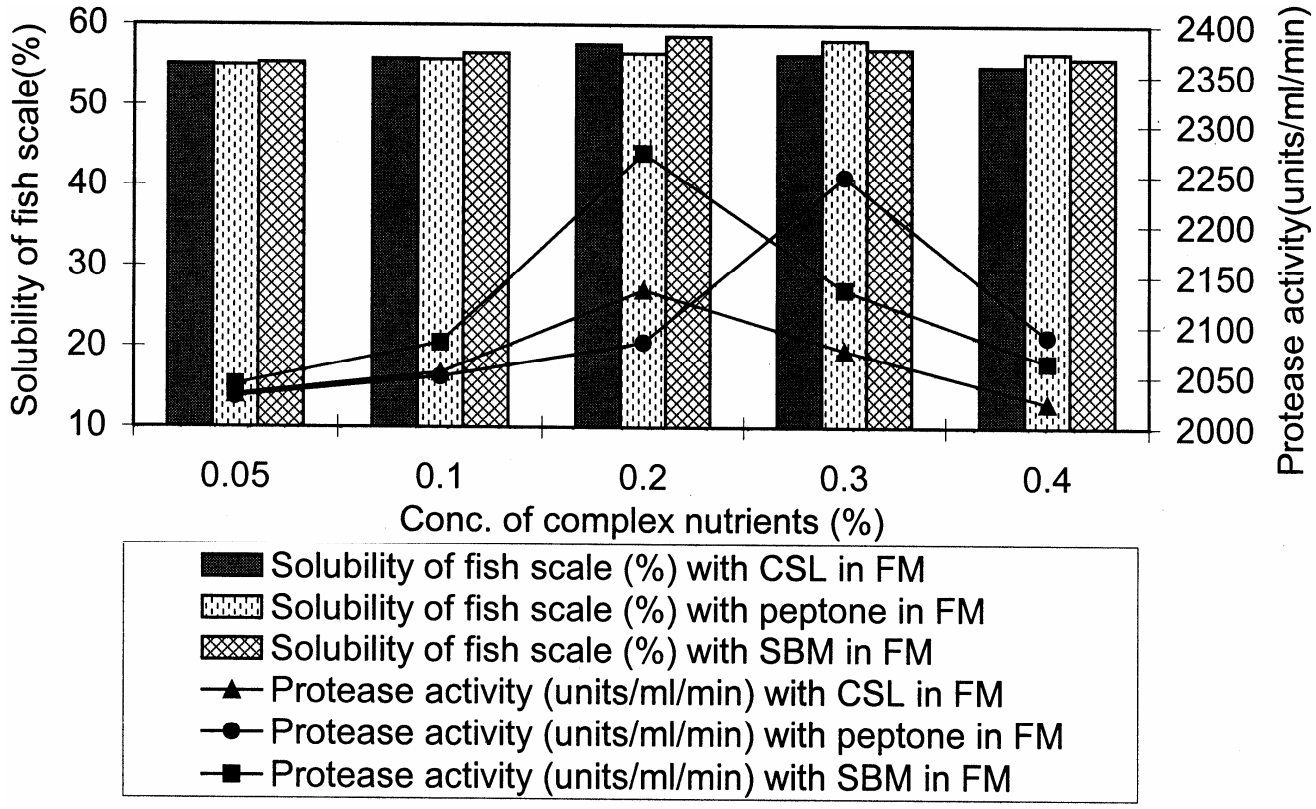

Fig. 3— Effect of corn-steep-liquor, peptone and soybean meal on protease production and solubilisation of fish scale by *A. niger* AB_{100}

Effects of Complex Nutrients

Eight different complex nutrients [peptone, extracts of yeast, wheat–bran, rice-bran, beef and malt, paddy-soak-liquor, corn-steep-liquor (CSL) & soybean-meal (SBM)] were tested in a conc. of 0.05, 0.1, 0.2 and 0.3 per cent based on their solid content. CSL (0.2%), SBM (0.2%) and peptone (0.3%) exert a very strong stimulatory effect on protease yield as well as protein solubilisation (Fig. 3). SBM (0.2%) was found to have maximum stimulatory effect on protease production ($p<0.001$).

Results from present study are supported by previous reports[41] where CSL was suitable nitrogen source for alkaline protease production and used as a
cheap source of nitrogen[42,43]. Besides nitrogen, CSL also provides several micronutrients, vitamins and growth promoting factor[14]. SBM was also reported to be a cheap and suitable nitrogen source during feather degradation[44], alkaline elastase[45] and extracellular alkaline proteinase production[46]. Peptone, which has a positive effect on protease production in this study, was reported to be a key nutrient material for alkaline protease synthesis[47,48].

Conclusions

Optimizing the requirements of inorganic nutrients, metal ions and various cost-effective substrates, a higher amount of solubilised protein can be recovered from the fish scale of *L rohita* by proteolytic activity of *A. niger* AB_{100}.

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