

## Full Length Research Paper

# The use of an economical medium for the production of alkaline serine proteases by *Bacillus licheniformis* NH1

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The present study is concerned with the selection of new economical media based on agricultural and marine-processing by-products for the production of alkaline proteases by *Bacillus licheniformis* NH1. Powders from different fish species were prepared and then tested as growth media at a concentration of 10 g/l for proteases production by NH1 strain. Powder prepared from whole Sardinelle was found to be the best substrate for the production of the alkaline protease. The NH1 strain exhibited a slightly greater protease production (2927 U/ml) in medium containing only whole Sardinelle powder than that obtained in control medium (2800 U/ml). Proteases were produced even when strain NH1 was cultivated in medium containing only powder prepared from combined heads and viscera Sardinelle (CHVSP), about 2117 U/ml. Protease production was also carried out in media containing hulled grain of wheat, a by-product of semolina factories, as carbon source. Maximum activity (2517 U/ml) was achieved when the strain was grown in medium containing hulled grain of wheat (10 g/l), casein peptone (2 g/l),  $K_2HPO_4$  (0.5 g/l) and  $KH_2PO_4$  (0.5 g/l). Moreover, protease production was considerably enhanced when the strain was grown in medium containing both hulled grain of wheat and CHVSP as carbon and nitrogen sources, respectively, (4771 U/ml). The study shows that hulled grain of wheat and powders from fishery by-products could be utilized as bacterial substrates for the production of alkaline proteases by *B. licheniformis* NH1.

**Key words:** Fish powder, hulled grain of wheat, heads and viscera, alkaline protease, *Bacillus licheniformis* NH1.

## INTRODUCTION

The expansion of biotechnology has created an increasing demand for new and low cost microbial growth substrate. In most instances, the growth medium accounts for approximately 40% of the production cost of industrial enzymes (Joo and Chang, 2005). Considering this fact, the use of cost-effective growth substrate for the production of alkaline proteases can significantly reduce the cost of

protease production.

Proteases are the most important industrial enzymes accounting for approximately 60% of the total industrial enzyme market (Rao et al., 1998). They have diverse applications in a wide variety of industries, such as in detergent, food, pharmaceutical, leather, silk and for recovery of silver from used X-ray films (Kumar and Takagi, 1999; Gupta et al., 2002). Of these, alkaline proteases are particularly important because they are both stable and active at high pH solutions and in the presence of surfactants and oxidizing agents (Gupta et al., 1999; Gupta et al., 2002). Their major application is in detergent industry, because the pH of laundry detergents is generally in the range of 9.0 - 12.0. Alkaline proteases are used as cleaning additives in detergents to facilitate the release of proteins strain. Proteases in detergent

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**Abbreviation:** CHVSP, Combined heads and viscera Sardinelle powder; LB, Luria-Bertani; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; MSP, meat Sardinelle powder; WSP, whole Sardinelle powder.

industries account for approximately 30% of the total world enzyme production (Kalisz, 1988). They are produced by a wide range of microorganisms including bacteria, moulds, yeasts and also mammalian tissues. Most of the commercial alkaline proteases were isolated from *Bacillus* species (Maurer, 2004).

Several studies have been undertaken to define culturing and nutritional conditions for obtaining higher yields of proteases. Extracellular production of proteases by microorganisms is greatly influenced by physical factors such as pH, temperature and incubation time, and by the composition of the medium (Johnvesly and Naik, 2001; Puri et al., 2002). Indeed, carbon and nitrogen sources were considered determinant factors (Frankena et al., 1986; Giesecke et al., 1991). Several studies have reported that proteins and peptides are necessary for effective protease production, while glucose repressed protease formation (Drucker, 1972; Ferrero et al., 1996; Fukushima et al., 1989). However, some works reported better protease synthesis in the presence of glucose as carbon source (Gessesse and Gashe, 1997; Mehrotra et al., 1999). Other medium compounds, such as metal ions and phosphorus source may also affect the amount of enzyme formation. Ghorbel-Frikha et al. (2005) reported the production of a calcium-dependent metalloprotease by *Bacillus cereus* BG1. The enzyme was detected only when the strain was cultivated in the presence of calcium. Protease production from *Bacillus* species using various agricultural residues (such as soybean meal, rice bran and wheat flour) was widely described in literature. Naidu and Devi (2005) reported the production of thermostable alkaline protease by *Bacillus* spp. K30 utilizing rice bran. Joo and Chang (2005) showed that maximum protease synthesis by alkalophilic *Bacillus* spp. I-312 was obtained when the bacterium was grown in a medium containing wheat flower and soybean meal as carbon and nitrogen sources, respectively. Soybean meal was also used as a nitrogen source for protease production by *Bacillus* spp. L21 in low cost producing media (Tari et al., 2006).

In Tunisia, semolina factories and fishery industries produce large quantities of by-products. Hulled grain of wheat, a by-product of semolina factories, constitutes a good source of starch at present utilized as a food material. Several reports showed also that hulled grain of wheat might be used as carbon source for metabolite production (Zouari et al., 2002). During fish processing, large quantities of waste, including heads and viscera are generated and discarded. They represent about 30% of the original raw material. These wastes, which represent an environmental problem to the fishing industry, constitute an important source of protein. Traditionally, these materials have been converted to powdered fish flour used as animal feed (Strom and Eggum, 1981). Since they are rich in proteins, fishes constitute a potential source of industrial peptones for a wide range of applications, especially as nitrogen and/or carbon source for microbiological use. However, few works have been reported concerning the use of non-hydrolysed fish

proteins for the growth and the production of metabolites by microorganisms (Ellouz et al., 2001).

In a previous search for alkaline protease-producing strains, which have potential industrial applications, *B. licheniformis* NH1 strain, producing alkaline proteases, was isolated from an activated sludge reactor treating fishery wastewaters (El Hadj-Ali et al., 2007). The crude extracellular protease produced by the isolate had optimal activity at 65-70°C and pH 10.0-11.0. The crude enzyme showed excellent stability and compatibility with many detergents tested. The strain exhibited higher productivity of alkaline protease in medium containing casein as carbon source (2800 U/ml). It showed higher growth but lower protease on starch. However, glucose repressed protease formation. The ability of NH1 strain to grow and produce alkaline proteases on casein as a substrate led us to look for the formulation of media based on cheap substrates.

The aim of this study was to examine the possible utilization of both fish powders and hulled grain of wheat as bacterial growth substrates for alkaline protease production by *B. licheniformis* NH1.

## MATERIALS AND METHODS

### Materials

Whole fish powders were prepared from different species of fish: Sardinelle (*Sardinella aurita*), Tub gurnard (*Trigla lucerna*), Common Pandora (*Pagellus erythrinus*), Red mullet (*Mullus barbatus*) and Salema (*Sarpa salpa*). Fish species were purchased from the local market at Sfax city Tunisia.

Casein sodium salt from bovine milk was purchased from Sigma Chemical Co. (St Louis, MO, USA). Casein peptone and yeast extract were from Bio-Rad (France). Trichloroacetic acid was from Carlo Erba Reactifs. Other chemicals and reagents used were of analytical grade.

### Bacterial strain

The microorganism used in this study was isolated from an activated sludge reactor treating fishery wastewaters. It was identified as *B. licheniformis* NH1 (El Hadj-Ali et al., 2007).

### Cultivation and culture conditions

Inocula were routinely grown in Luria-Bertani (LB) broth medium composed of peptone (10 g/l), yeast extract (5 g/l) and NaCl (5 g/l) (Miller, 1972). The previously optimized M1 medium used for protease production was composed of casein (5 g/l), yeast extract (2 g/l), K<sub>2</sub>HPO<sub>4</sub> (0.5 g/l), KH<sub>2</sub>PO<sub>4</sub> (0.5 g/l) and KCl (1.5 g/l) at pH 8.0 (El Hadj-Ali et al., 2007). The fish medium used for protease production was composed of only fish powder (10 g/l). The initial M2 hulled grain of wheat medium was composed of 10 g/l hulled grain of wheat, 2 g/l yeast extract, 0.5 g/l K<sub>2</sub>HPO<sub>4</sub> and 0.5 g/l KH<sub>2</sub>PO<sub>4</sub> at pH 8.0. Media were autoclaved at 120°C for 20 min. Hulled grain of wheat contains 50 - 60% starch, 8 - 12% proteins, 1.5% cellulose and 5% carbohydrates.

Cultivations were conducted in 25 ml of medium in 250 ml conical flasks maintained at 37°C. Incubation was carried out with agitation at 200 rpm for 24 h. The cultures were centrifuged and the supernatants were used for estimation of proteolytic activity.

**Table 1.** Chemical composition of fish powders (g/100 g product).

Fish powder	Water	Proteins	Ash	Lipids	Cl	Na	K	Ca
WSP	5.6	62.4	10.6	19.3	0.174	0.427	0.362	1.805
Tub gurnard	5.4	63.6	17.9	12.4	0.083	0.484	0.452	3.883
Common pandora	5.9	52.8	19.5	20.3	0.084	0.472	0.458	5.146
Red mullet	5.4	59.8	10.8	21.2	0.102	0.427	0.362	1.975
Salema	6.4	64.4	19.4	9	0.091	0.402	0.278	3.905
MSP	1.2	76.2	4.5	15.8	0.34	0.51	0.18	0.47
CHVSP	1.3	48.6	20.9	26.9	0.19	0.49	0.38	0.79

WSP: Whole Sardinelle powder; MSP: meat Sardinelle powder; CHVSP: combined heads and viscera Sardinelle powder.

### Cell growth determination

The growth of the microorganism was estimated by the determination of colony forming units (CFU/ml). All experiments were carried out in duplicate and repeated at least twice.

### Preparation of fish powders

To obtain fish-meat powders, heads and viscera were first eliminated. The raw material was then heated till boiling. The bones were removed and the cooked meat was pressed to remove water and fat. The resulting pressed product was minced in a meat grinder and then dried at 80°C for 24-48 h. The dried fish preparation was minced again to obtain a fine powder and then stored in glass bottles at room temperature. In order to obtain whole fish or combined heads and viscera fish powders, raw materials were cooked, pressed, minced and then dried.

### Chemical composition

Dry weight of fish powders was determined after heating samples at 105°C to constant weight and ash content was determined after heating dried samples at 600°C for 4 h. Total nitrogen content was determined using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor 6.25. Crude fat was determined gravimetrically after Soxhlet extraction of dried samples with diethyl ether.

### Assay of proteolytic activity

Protease activity was measured by the method of Kembhavi et al. (1993) using casein as a substrate. A 0.5 ml aliquot of the culture supernatant, suitably diluted, was mixed with 0.5 ml 100 mM glycine-NaOH (pH 10.0) containing 1% (w/v) casein and incubated for 15 min at 70°C. The reaction was stopped by addition of 0.5 ml trichloroacetic acid (20%, w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000 g for 15 min to remove the precipitate. The acid soluble material was estimated spectrophotometrically at 280 nm. A standard curve was generated using solutions of 0 - 50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min under the experimental conditions used.

Protease activities represent the means of at least two determinations carried out in duplicate. The difference between values did not exceed 5%.

### Detection of enzyme activity by zymography

Protease activity staining was performed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Garcia-Carreno et al. (1993) with slight modification. The sample was not heated before electrophoresis. After electrophoresis, the gel was submerged in 100 mM glycine-NaOH buffer (pH 10.0) containing 2.5% Triton X-100, with shaking for 30 min to remove SDS. Triton X-100 was removed by washing the gel three times with 100 mM glycine-NaOH buffer (pH 10.0). The gel was then incubated with 1% (w/v) casein in 100 mM glycine-NaOH buffer (pH 10.0) for 40 min at 50°C. Finally, the gel was stained with 0.25% Coomassie Brilliant Blue R250 in 45% ethanol-10% acetic acid and destained with 5% ethanol-7.5% acetic acid. The development of clear zones on the blue background of the gel indicated the presence of protease activity.

## RESULTS AND DISCUSSION

### Preparation of fish substrates

Fish powders were prepared from different species of fish and used as growth media for protease production by NH1 strain. Moreover, fish powders were prepared from meat Sardinelle (MSP) and combined heads and viscera Sardinelle (CHVSP). The main chemical composition of fish powders is given in Table 1. These data show that fish powders are rich in both organic and inorganic materials. Notably, fish powders contain the essential substances required in microbial media, such as sources of carbon, nitrogen and minerals. As shown in Table 1, all whole fish powders contained high protein content (between 52.8 and 64.4%, by-weight). MSP contained high protein content (76.2%) but lower ash and a relatively high lipid content. CHVSP contained more lipids and less protein content than whole and meat Sardinelle powders and due to higher bone content there are more minerals.

### Protease production by *B. licheniformis* NH1 strain grown on different fish powders

In order to test if fish preparations promote biomass and

**Table 2.** Effect of different whole fish powders on growth and protease production by *B. licheniformis* NH1.

Fish powder	Protease activity (U/ml)	Biomass (x 10 <sup>6</sup> UFC/ml)
Control (M1)	2800	80
Sardinelle	2927	225
Tub gurnard	2487	580
Common pandora	2418	520
Red mullet	1756	105
Salema	1647	145

Cultivations were performed for 24 h at 37°C in media containing only whole fish powder at a concentration of 10 g/l. Values are means of three independent experiments: standard deviations  $\pm$  2.5% (based on three replicates). M1 medium consist of 5 g/l casein, 2 g/l yeast extract, 0.5 g/l K<sub>2</sub>HPO<sub>4</sub>, 0.5 g/l KH<sub>2</sub>PO<sub>4</sub> and 1.5 g/l KCl.

**Table 3.** Effect of WSP concentrations on growth and protease production by *B. licheniformis* NH1.

Parameters	WSP (g/l)					
	2	5	7.5	10	15	20
Protease activity (U/ml)	633	1891	2967	3018	3331	3382
Biomass (x 10 <sup>6</sup> UFC/ml)	8	34	180	230	420	900
Yield (10 <sup>3</sup> U/g substrate)	316.5	378.2	395.6	301.8	222	169.1

Cultivations were performed for 24 h at 37°C in media containing different concentration of whole Sardinelle powder. Values are means of three independent experiments: standard deviations  $\pm$  2.5% (based on three replicates).

enzyme synthesis, protease production by *B. licheniformis* NH1 was assayed in media containing only whole fish substrates at a concentration of 10 g/l and compared with the control medium (M1) containing casein and yeast extract as sources of carbon and nitrogen, respectively. As shown in Table 2, all the whole fish substrates tested enhanced growth and protease production by NH1 strain, indicating that it was not necessary to add ingredients (such as salts and yeast extract) to fish medium. Maximum activity was obtained with whole Sardinelle powder (WSP) (2927 U/ml) and it was slightly higher to that obtained in control medium M1 (2800 U/ml) (El Hadj-Ali et al., 2007). However, lower biomass and protease activity were obtained with whole Red mullet and whole Salema powders (1700 U/ml).

Since whole Sardinelle preparation was the best substrate for protease synthesis by NH1 strain, the effects of its various concentrations on enzyme production was studied. As shown in Table 3, the highest protease production (3000-3300U/ml) was achieved between 7.5 and 20g/l. However, maximum protease yield was obtained with 7.5 g/l of WSP.

#### Protease production by *B. licheniformis* NH1 strain grown on hulled grain of wheat based medium

Since hulled grain of wheat is an inexpensive and readily

available substrate, it was to be a possible candidate for the cost-effective production of extracellular proteases. So the capacity of protease production by NH1 strain was studied in medium M2 containing different concentrations of hulled grain of wheat as carbon substrate. As shown in Table 4, protease activity increased with increasing hulled grain of wheat concentration and reached a value of about 2000 U/ml with 10 g/l. Beyond 10 g/l, the level of protease activity did not vary significantly. The yield, estimated as protease produced per g of substrate used, reached a maximum value of about 200.4 x 10<sup>3</sup>U/g substrate, at 10 g/l of hulled grain of wheat.

The effect of different organic (casein peptone and yeast extract) and inorganic (ammonium sulphate) nitrogen sources on protease synthesis in M2 medium containing hulled grain of wheat at 10 g/l, K<sub>2</sub>HPO<sub>4</sub> 0.5 g/l and KH<sub>2</sub>PO<sub>4</sub> 0.5 g/l was also investigated. As shown in Table 5, the best nitrogen source for protease production was casein peptone (2520 U/ml) followed by yeast extract (2003 U/ml). However, enzyme production was significantly reduced with ammonium sulphate as nitrogen source.

Conflicting results regarding the effects of organic and inorganic nitrogen sources on alkaline protease production by *Bacillus* sp. have been reported in the literature. Do Nascimento and Martins (2004) reported maximum enzyme activity by thermophilic *Bacillus* sp. SMIA-2 with

**Table 4.** Effect of hulled grain of wheat concentration on growth and protease production by NH1 strain.

Parameters	Hulled grain of wheat (g/l)				
	2	5	10	15	20
Protease activity (U/ml)	585	995	2004	2142	2230
Biomass (x 10 <sup>7</sup> UFC/ml)	37	42	55	170	193
Yield (10 <sup>3</sup> U/g substrate)	292.7	199	200.4	142.8	111.5

Cultivations were performed for 24 h at 37°C in media consisting of (g/l): yeast extract 2, K<sub>2</sub>HPO<sub>4</sub> 0.5, KH<sub>2</sub>PO<sub>4</sub> 0.5 and different concentrations of hulled grain of wheat. Values are means of three independent experiments: standard deviations ± 2.5% (based on three replicates).

**Table 5.** Effect of different nitrogen sources (2 g/l) on protease and biomass production by *B. licheniformis* NH1.

Parameters	Nitrogen source		
	Casein peptone	Yeast extract	Ammonium sulphate
Protease activity (U/ml)	2520	2003	291
Biomass (x 10 <sup>7</sup> UFC/ml)	122	53	22

Cultivations were performed for 24 h at 37 °C in media consisting of (g/l): hulled grain of wheat 10, nitrogen source 2, K<sub>2</sub>HPO<sub>4</sub> 0.5 and KH<sub>2</sub>PO<sub>4</sub> 0.5. Values are means of three independent experiments: standard deviations ± 2.5% (based on three replicates).

**Table 6.** Effect of casein peptone concentration on growth and protease production by *B. licheniformis* NH1.

Parameters	Casein peptone (g/l)				
	0	0.5	1	2	5
Protease activity (U/ml)	600	2033	2116	2517	1764
Biomass (x 10 <sup>7</sup> UFC/ml)	32	50	53	122	33

Cultivations were performed for 24 h at 37°C in media consisting of hulled grain of wheat 10 g/l, K<sub>2</sub>HPO<sub>4</sub> 0.5 g/l, KH<sub>2</sub>PO<sub>4</sub> 0.5 g/l and different concentrations of casein peptone. Values are means of three independent experiments: standard deviations ± 2.5% (based on three replicates)

ammonium nitrate as inorganic nitrogen source and found that protease production was repressed by organic nitrogen sources, while other works reported a higher protease production with organic nitrogen source (Feng et al., 2001; Patel et al., 2005).

Based on these observations, casein peptone was selected and its various concentrations were tested for the production of protease. As shown in Table 6, the enrichment of growth medium with casein peptone enhanced significantly both bacterial growth and protease production. Maximum activity was achieved at a concentration of 2g/l (2517 U/ml); further additions of casein peptone decreased biomass and protease synthesis. Protease synthesis was significantly low in the absence of casein peptone (600 U/ml).

#### Protease production in medium containing both fish powder and hulled grain of wheat

The ability of NH1 strain to grow and produce alkaline protease in media containing fish powders or hulled grain

of wheat, led us to study the formulation of a new medium based on these substrates. Protease synthesis was investigated in M2 medium containing hulled grain of wheat (10 g/l) as carbon source and different concentrations of CHVSP as nitrogen source. As shown in Table 7, protease production was significantly low with CHVSP up to 2 g/l. Maximum activity was observed with 10 g/l, about 4771 U/ml, which was about 8-fold over the medium without or containing 2 g/l CHVSP. Beyond 10 g/l of CHVSP, protease activity decreased while biomass increased.

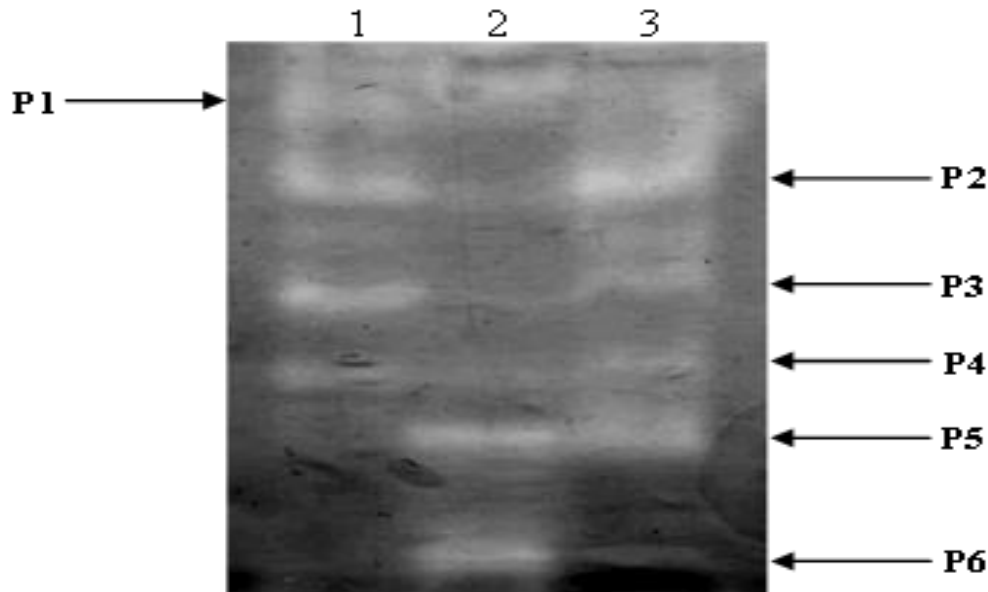
#### Comparison of extracellular proteases production on various media

Many enzymes, such as proteases and α-amylases, have been proven to be renaturable after electrophoresis in the presence of SDS (Lacks and Springhorn, 1980). In this study, zymography, a sensitive and rapid assay method, was used for analyzing enzyme activity. Proteolytic activity of the *B. licheniformis* NH1 crude enzyme grown

**Table 7.** Effect of CHVSP concentration on growth and protease production by *B. licheniformis* NH1.

Parameters	CHVSP (g/l)						
	0	2	5	7	10	15	20
Protease activity (U/ml)	600	600	2094	3258	4771	4124	3738
Biomass (UFC/ml)	285 10 <sup>6</sup>	250 10 <sup>6</sup>	240 10 <sup>7</sup>	257 10 <sup>8</sup>	48 10 <sup>9</sup>	85 10 <sup>9</sup>	126 10 <sup>9</sup>

Cultivations were performed for 24 h at 37°C in media consisting of hulled grain of wheat (10 g/l), K<sub>2</sub>HPO<sub>4</sub> (0.5 g/l), KH<sub>2</sub>PO<sub>4</sub> (0.5 g/l) and different concentrations of CHVSP. Values are means of three independent experiments: standard deviations  $\pm$  2.5% (based on three replicates).



**Figure 1.** Zymogram analysis of proteases excreted by *B. licheniformis* NH1 on various media. (1) Culture supernatant obtained on casein, (2) gruel, and (3) WSP based medium.

on casein, gruel and WSP based medium was studied using casein as substrate.

As shown in Figure 1, the crude protease preparation showed multiple types of proteases and their number depends on the composition of culture medium. When NH1 strain was grown in the presence of casein, at least four proteases were induced (P1, P2, P3 and P4). At least four major clear bands of proteolytic activity (P2, P3, P4 and P5) were detected when the strain was cultured on WSP based medium. While the culture supernatant produced in the presence of gruel showed only two proteases (P5 and P6). The induction of P5 and P6 proteases could be explained by the fact that complex substrates as gruel and WSP require others types of proteases to be assimilated by NH1 strain.

Ferrero et al. (1996) also reported that *B. licheniformis* MIR 29 produced two alkaline serine proteases with molecular masses of 25 and 40 kDa. Manachini and Fortina (1998) reported the production of three proteases, by a halotolerant strain of *B. licheniformis*, with molecular mass of 15, 22.5 and 33 kDa.

The purification of different proteases produced by NH1

strain is now under investigation in our laboratory.

## Conclusion

In this study, the production of alkaline protease by *B. licheniformis* NH1 grown in economical medium was investigated. The NH1 strain produced maximum protease in WSP (2927 U/ml), followed by whole Tub gurnard (2487 U/ml) and Common pandora (2418 U/ml). Moreover, the strain was found to grow well and produce higher protease activity with CHVSP, although this powder contains low protein content. The enhancement of protease activity in media containing only CHVSP may be due to the presence in fish powders of bioactive substances acting as protease inducers.

Although, hulled grain of wheat contains protein (8-12%), supplementing it with organic nitrogen source lead to an increase in protease activity. Among various nitrogen sources studied, the NH1 strain produced maximum protease with casein peptone (2520 U/ml) followed by yeast extract (2003 U/ml). Combination of hulled grain of

wheat and fish powders enhanced significantly the protease activity (4771 U/ml) with comparison with commercial substrates (2800 U/ml). According to optimization experiments, the optimum protease medium formulated contained hulled grain of wheat (10 g/l), CHVSP (10 g/l), K<sub>2</sub>HPO<sub>4</sub> (0.5 g/l) and KH<sub>2</sub>PO<sub>4</sub> (0.5 g/l).

The use of these cheaper and readily available sources of both carbon and nitrogen sources instead of commercial substrates are the key attraction for the cost-effective production of an extracellular proteases. Additional experiments should be conducted to elucidate the behavior of the strain with each fish powder studied and to identify bioactive substances or inducers present in fish powder.

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