Scale-Up of an Alkaline Protease from *Bacillus pumilus* MTCC 7514 Utilizing Fish Meal as a Sole Source of Nutrients

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Fish meal grades SL1 and SL2 from Sardine (*Sardinella longiceps*) and NJ from Pink Perch (*Nemipterus japonicas*) were evaluated as a sole source of carbon and nitrogen in the medium for alkaline protease production by *Bacillus pumilus* MTCC 7514. The analysis of the fish meal suggests that the carbon and nitrogen contents in fish meal are sufficient to justify its choice as replacement for other nutrients. Protease production increased significantly (4,914 U/ml) in medium containing only fish meal, compared with the basal medium (2,646 U/ml). However, the elimination of inorganic salts from media reduced the protease productivity. In addition, all the three grades of fish meal yielded almost the same amounts of protease when employed as the sole source of carbon and nitrogen. Nevertheless, the best results were observed in fish meal SL1 medium. Furthermore, protease production was enhanced to 6,966 U/ml and 7,047 U/ml on scaling up from flask (4,914 U/ml) to 3.7 and 20 L fermenters, respectively, using fish meal (10 g/l). Similarly, the corresponding improvement in productivities over flask (102.38 U/ml/h) was 193.5 and 195.75 U/ml/h in 3.7 and 20 L fermenters, respectively. The crude protease was found to have dehairing ability in leather processing, which is bound to have great environmental benefits.

**Keywords:** Fish meal, protease, dehairing, scale-up, fermenters

The increasing use of microbial proteases in different industries has created an increasing demand of new and low-cost raw material sources for fermentation. In most instances, the growth/production medium accounts for approximately 40% of the production cost of industrial enzymes [6]. It is therefore imperative that the medium cost be as low as possible for the fermentation to be economically viable. Using fish meal from the fish processing industry, which is a rich source of protein (over 50–60%), might contribute to a reduction in production cost of protease at commercial scales. Fish meal is one of the main products obtained from fish waste, by-catch, and other species [1], and it is mostly used as an ingredient in food for fish, poultry, and crustaceans. It is a complex mixture of proteins and fatty acids, and a rich source of minerals such as calcium and phosphorus as inorganic compounds, and is readily available throughout India. It is generally produced from fish that are not ideal for human consumption, by subjecting it to steam treatment and separating the resulting fish liquor (contains the fish oil) and solid matter, which is further dried to obtain fish meal (http://rajfishmeal.com/products.htm). Its affordable cost and nutrient profile (http://www.fao.org/ag/aga/agap/frg/afris/data/332.htm) render it an ideal substrate for microbial protease production. Some reports have shown the utilization of fish processing by-products like sardinella heads and viscera flour [2, 7], underutilized fish such as *Johnius dissimueri* [16], cuttlefish (*Sepia officinalis*) by-products powder (CFP) [15], and shrimp and crab shell powder [17] as substrates for protease production by bacteria/fungi. There are no reports on protease fermentation using fish meal as a sole source of carbon and nitrogen.

The aims of this study were to evaluate the three grades of fish meal as a sole substrate to replace a relatively expensive medium, for protease production from *Bacillus pumilus* MTCC 7514 in flasks as well as in laboratory fermenters, and to evaluate the enzyme’s efficacy in the unhairing of goat skins.

**Materials and Methods**

**Substrate and Chemicals**

Fish meals used in this study were sourced from Raj Fish Meal & Oil Company, Malpe and Asian Fish Meal & Oil Company, Mangalore...
Table 1. Chemical composition of fish meal.

<table>
<thead>
<tr>
<th>Composition</th>
<th>SL1 (% w/w)</th>
<th>SL2 (% w/w)</th>
<th>NJ (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%) max</td>
<td>7–10</td>
<td>11</td>
<td>7–10</td>
</tr>
<tr>
<td>Total ash (%) max</td>
<td>22–25</td>
<td>18</td>
<td>22–28</td>
</tr>
<tr>
<td>Fat (%) max</td>
<td>10–12</td>
<td>10</td>
<td>8–10</td>
</tr>
<tr>
<td>Protein (% min)</td>
<td>58–60</td>
<td>65</td>
<td>50–55</td>
</tr>
<tr>
<td>Sand (%) max</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Salt (%) max</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

both in Karnataka State, India. Laboratory-grade tryptone, agar, maltodextrin, yeast extract, and skim milk were purchased from Hi-Media, India. All other chemicals used were of analytical grade. The water for the experiments was demineralized in an ion-exchange unit before use.

Microorganism and Inoculum

Bacillus pumilus MTCC 7514, isolated earlier in our laboratory from a marine soil sample, was employed in the present study. The inoculum was prepared by transferring a loopful of culture from nutrient agar slants to 250 ml Erlenmeyer flasks containing 50 ml of LB (Luria–Bertani) medium and incubating at 30°C in a shaker (Orbitek, Scigenics Biotech, India) at 120 rpm for 20 h. The resulting active culture at a concentration of 2% (v/v) was used to inoculate the production media in the flasks.

Medium for Protease Production

The previously optimized basal medium [11] was used for protease production and was composed of (g/l): maltodextrin, 5; yeast extract, 5; skim milk, 5; NaCl, 1.2; MgSO$_4$$\cdot$7H$_2$O, 0.6; KH$_2$PO$_4$, 0.6, and CaCl$_2$$\cdot$2H$_2$O, 0.3. Inorganic components were dissolved separately in water, autoclaved, and then added to the medium aseptically. Tris buffer (0.1 M) was added to the production medium to adjust the initial pH to 7.5. This medium was used as the control for comparison of protease production throughout.

Enzyme Assay

Proteolytic activity of the enzyme was determined by the method of Kunitz [9]. In brief, 0.1 ml of culture supernatant was incubated with 1.9 ml of casein solution [1% (w/v) Hammerstein casein in 0.05 M carbonate buffer, pH 9.5] for 10 min at 60°C, and then 3.0 ml of trichloroacetic acid [5% (w/v)] was added to terminate the reaction. The reaction mixture was then filtered through Sonar filter paper and the released tyrosine content was determined spectrophotometrically at 280 nm (Shimadzu UV-2401). One unit of enzyme activity is defined as the amount of enzyme required to release 1 µg of tyrosine/ml/min under standard assay conditions.

Analysis of Fish Meal

The composition of fish meal as provided by the suppliers is summarized in Table 1. Elemental analysis of fish meal was performed using a CHNS analyzer (Elementor, Germany). About 3 mg of the sample was packed in a silver boat and placed in a CHNS analyzer where it was converted to elements by heating at 1,150°C and further oxidized to subsequent compounds and estimated (Table 2).

Table 2. Elemental composition of fish meal.

<table>
<thead>
<tr>
<th>Element</th>
<th>SL1 (% w/w)</th>
<th>SL2 (% w/w)</th>
<th>NJ (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total carbon</td>
<td>37.5</td>
<td>36.1</td>
<td>38.2</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>9.89</td>
<td>10.14</td>
<td>9.24</td>
</tr>
<tr>
<td>Total hydrogen</td>
<td>5.64</td>
<td>5.83</td>
<td>6.1</td>
</tr>
<tr>
<td>Total sulfur</td>
<td>0.71</td>
<td>0.70</td>
<td>0.52</td>
</tr>
<tr>
<td>Total oxygen</td>
<td>22.3</td>
<td>22.8</td>
<td>21.9</td>
</tr>
</tbody>
</table>

SL1 and SL2 fish meals were processed from Sardine (Sardinella longiceps) fish, and the NJ specimen was processed from Pink Perch (Nemipterus japonicus).

Evaluation of Fish Meal as Sole Substrate

The components of the basal medium for optimum production of the protease were replaced with fish meal sequentially. Yeast extract and skim milk were replaced one by one with fish meal (5, 10, and 20 g/l). Subsequently, the effect of maltodextrin at concentrations 2 and 5 g/l in fish meal media was studied over time. In addition, protease production in fish meal media with and without supplementation of inorganic salts was evaluated at the same concentrations as used in basal media (control). The best of the three grades followed by its optimum concentration was determined.

Scale-Up of Protease Production in Fermenters

Protease production was scaled-up from flask to 3.7 L fermenter (Bioengineering KLF) with a working volume of 2.5 L using optimized fish meal medium. The medium was sterilized in situ at 121°C for 20 min and inoculated with 100 ml [4.0% (v/v)] of 20-h-old culture. Inorganic salts were autoclaved separately and transferred to the fermenter aseptically. The agitation speed was set to 300 rpm and compressed air was sparged at a flow rate of 1.0 vvm. Initial pH of the fish meal medium was adjusted to 7.0 using 0.1 N NaOH and was monitored but not controlled during fermentation. Hi-Media silicon oil [10% (v/v) in distilled water] was used as antifoam to control foaming during fermentation. Samples were collected at regular intervals of 6 h, and subsequently the protease activity and biomass were estimated. Biomass was estimated by the determination of colony forming unit (CFU) by the serial dilution method [12]. All experiments were carried out in duplicate. The same study was carried out in a 20 L fermenter (Bioengineering L1523) with working volume of 14 L and all other conditions remaining the same.

Crude Protease for Dehairing of Goat Skin

A fresh goat skin weighing 1.32 kg was procured from a local meat shop. It was washed properly and kept for soaking in about 200% (w/w) water for 2 h, containing 0.1% (w/v) Bronopol and 0.2% (w/v) sodium benzoate. The soaking pH was adjusted to 10.0 with sodium carbonate (q.s.). The goat skin was cut into three pieces, including one small piece serving as a control. Dehairing was carried out by the dip method employing 150% (w/w) cell-free crude fermentation broth just sufficient to immerse the skin at 30°C. Dehairing of skin pieces was observed at different time intervals as described by Huang et al. [5].
RESULTS AND DISCUSSION

Evaluation of Fish Meal (FM) SL1 as Sole Substrate for the Protease Production

The basal medium used as the control had been previously optimized by Prabhawati [11]. In this study, the focus was to first evaluate fish meal as a source of nitrogen in order to replace the yeast extract used in basal medium, which is comparatively costlier. The C/N ratio for fish meals was found to be 3–4 (Table 1), which is similar to the C/N ratio of animal fleshing reported by Kumar et al. [8] for use as a sole substrate in medium for protease production. Fig. 1 shows the protease production in the medium containing fish meal as a nitrogen source replacing yeast extract. Protease production was higher in the medium containing fish meal at 10 g/l and 20 g/l, whereas production at 5 g/l fish meal concentration was lower and similar to the control medium; it indicates that fish meal is a better nitrogen source. When both yeast extract and skim milk were replaced with different concentrations of fish meal, 10 g/l gave the highest activity at 36 h, indicating that fish meal alone is a better nitrogen source as well as a good inducer. Initially, production was slow at 20 g/l concentration but gradually increased until 60 h, thereafter stabilizing.

The apparent lag followed by a slow increase in protease production may be the result of substrate inhibition (Fig. 2). The production profile at 20 g/l concentration was similar to the production in positive control which is the medium indicated in Fig. 1 (FM 20 g/l without yeast extract). Since yield and productivity were much higher at 10 g/l of fish meal, it was selected as the optimum concentration for protease production in this test. Next, the effect of elimination of maltodextrin (carbon source) was evaluated. Fig. 3 shows that protease production was almost equal in the positive control (FM 10 g/l without yeast extract and skim milk) medium as well as in the absence of maltodextrin, indicating that fish meal itself can be used as a carbon and nitrogen source as well as an inducer as it contains 58–60% protein and 10–12% fat (Table 1). It is quite probable that the fat content in fish meal is supporting the organism for its carbon and energy requirements. However, the monitoring of the fat content was not attempted in this study. The increase in protease production in fish meal media might be due to bioactive molecules present in protein fractions and fat contents, as reported by Ellouz et al. [2], as well as...
high mineral content as represented by the ash value (Table 1), which varies from 18% to 28%. Next, the effect of inorganic salts on protease production in fish meal media was evaluated (Fig. 4). In the absence of inorganic salts in fish meal media, both the yield and productivity were lower, justifying the need for minerals in the medium for protease production.

Ellouz et al. [3] have studied the elimination of commercial nitrogen source by fish meat powder and reported the protease synthesis in medium containing fish meat powder to be higher (7,800 U/ml) than that obtained with peptone from casein (7,222 U/ml). In a similar report, Souissi et al. [15] have reported cuttlefish powder (CFP) from Sepia officinalis by-products as a fermentation substrate for microbial growth and protease production by several species of bacteria: Bacillus licheniformis, Bacillus subtilis, Pseudomonas aeruginosa, Bacillus cereus BG1, and Vibrio parahaemolyticus. All microorganisms grew well and produced protease when cultivated in medium containing only CFP, indicating that the strains can obtain their carbon and nitrogen source requirements directly from whole by-product proteins. It is to be reiterated that there are no reports with commercially marketed fish meal as a sole source of nutrients for protease fermentation.

### Protease Production in Different Grades of Fish Meal

Different grades of fish meal were evaluated to check their performance consistency of protease production by Bacillus pumilus MTCC 7514. SL1 and SL2 showed higher protease production of 4,914 U/ml and 4,779 U/ml, respectively, and also similar time profiles, whereas protease production in NJ fish meal was relatively low but was better when compared with control medium (Fig. 5). The reduced protease production in NJ fish meal medium may be due to the reduced protein content (Table 1), variation in amino acid concentration, and the digestibility of proteins, which may vary with the fish species used for the processing of fish meal [1]. Hadj-Ali et al. [4] have also reported sardinella fish powder as being the best substrate in medium for production of alkaline protease, among other fish powders tested.

### Protease Production in Different Concentrations of Fish Meal Alone

When the protease production was carried out with different concentrations of fish meal alone as a carbon and nitrogen source as well as inducer, as seen from Fig. 6, the maximum protease production was observed at the 36th h at 5 and 10 g/l concentrations of fish meal, whereas maximum protease production in control and fish meal media at 20 g/l concentration was obtained at the 48th h. The highest protease activity was at 20 g/l. However, 10 g/l concentration was selected as the optimum concentration for protease production since the yield and productivity were higher. Hadj-Ali et al. [4] have also studied the

### Table 3. Comparison (with published data) of protease production by fish by-products and fish meal fermentation.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Protease activity, U/l (× 10³)</th>
<th>Substrate supplied, g/l</th>
<th>Yield, U/g (× 10³)</th>
<th>Time of maximum protease production, h</th>
<th>Productivity, U/l/h (× 10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kamoun et al. [7]</td>
<td>8,473 (Flasks)</td>
<td>20</td>
<td>423.65</td>
<td>42</td>
<td>201</td>
</tr>
<tr>
<td>Ellouz et al. [3]</td>
<td>7,500 (Flasks)</td>
<td>10</td>
<td>780</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>Hadj-Ali et al. [4]</td>
<td>2,967 (Flasks)</td>
<td>7.5</td>
<td>395.6</td>
<td>24</td>
<td>123.6</td>
</tr>
<tr>
<td>Souissi et al. [15]</td>
<td>821 (Flasks)</td>
<td>10</td>
<td>82.1</td>
<td>24</td>
<td>34.2</td>
</tr>
<tr>
<td>This study</td>
<td>4,914 (Flasks)</td>
<td>10</td>
<td>491.4</td>
<td>48</td>
<td>102.38</td>
</tr>
<tr>
<td>This study</td>
<td>7,047 (Fermenters)</td>
<td>10</td>
<td>704.7</td>
<td>36</td>
<td>195.75</td>
</tr>
</tbody>
</table>
protease production in medium containing different concentrations of fish powder and reported that the production increased rapidly with increase in concentration up to 10 g/l, thereafter becoming insignificant. That the carbon and nitrogen were obtained from the fish meal alone is also supported by Kamoun et al. [7]. However, in their study, protease production by Bacillus cereus BG1 was 8,473 U/ml against 20 g/l substrate supplied, whereas our experiments resulted in 4,914 U/ml for 10 g/l substrate supplied, which translates to a yield coefficient that is higher by 16%. However, using 20 g/l fish meal in our studies gave a lower yield than Kamoun et al. [7]. Table 3 shows the comparison of our results with published data. In a similar study using fish waste preparation as sole fermentation substrate, Ellouz et al. [3] reported a high yield (780 × 10^3 U/g). However, there is no mention of the fermentation time, which makes it difficult to make a comparison. In addition, the strain used was Pseudomonas aeruginosa, deemed an opportunistic pathogen and unlikely to be used for commercial production without safety and protective measures. In another such study, whole fish powder was used as the sole substrate for fermentation by Bacillus licheniformis and a yield of 395 × 10^3 U/g was reported. Hadj-Ali et al. [4] and Souissi et al. [15] have reported a protease yield of 395.6 and 82.1 U/g, respectively, which is comparatively less when compared with the protease yield of our present study (Table 3).

**Upscaling Protease Production in Fermenters**

The handful of literature reports on protease production using fish powder/waste is confined to flask studies and there is no mention of any scale-up experiments using fermenters. For a better understanding of the potential of the system for technology capability, it is necessary to conduct experiments at gradually increasing scales. In this study, we have scaled-up protease production in 3.7 and 20 L fermenters using fish meal SL1 as a source of nutrients. Protease production started after the 12th h, reached a maximum at the 36th h, and declined thereafter. The maximum protease production was 6,966 and 7,047 U/ml in 3.7 and 20 L fermenters respectively, whereas at flask level, it was 4,833 U/ml (Fig. 7). The increase in production at the fermenter level is because of enhanced aeration and agitation, which are generally not optimal at the flask level. The DO (dissolved oxygen) and pH profiles in the medium during fermentation are given in Fig. 8, exhibiting a normal behaviour, and similar pH and DO profiles have been reported by Seeta Laxman et al. [13]. It appears that the enzyme production increases after biomass growth slows down from 18 h onwards (Fig. 7). There was a sharp decline in dissolved oxygen from the 6th to 12th h, which represents the accelerating growth phase. Singh et al. [14] have also reported a sharp decline in DO, when the fermentation was carried out at an agitation of 300 rpm and aeration of 1 vvm, which is similar to our study. In another study, Nadeem et al. [10] have reported a sharp decline in DO to zero from the 5th to 10th h, thereafter constant.

**Fig. 7.** Comparative study of protease production and biomass in fermenters. SL1 10 g/l with inorganic salts. PA, protease activity; BM, biomass.

**Fig. 8.** Variation of dissolved oxygen (DO) saturation and pH in fermenters.
until the 26th h, and increased when the fermentation was carried out at an agitation of 300 rpm and aeration of 1 vvm where the pH of the medium was controlled with NaOH/HCl. This is the first report for production of protease using fish meal or other fish-processing waste/by-products in fermenters.

Dehairing Application

Notwithstanding the lack of published data on fermenter studies, it is also to be mentioned that there are no reports either on exploring the potential of the protease from fish meal fermentation in some important application or indicating the actual cost benefit obtained by replacement with other nitrogen sources. Therefore, recognizing the need for filling this gap as well as considering the increasing interest in enzymatic dehairing of skins and hides (leather processing), a preliminary dehairing study was carried out with the crude protease cell-free fermentation broth. Fig. 9 (A–C) show photographs of the untreated skin and dehaired pelts. Dehairing was observed with both concentrations (crude and twice diluted) of enzyme solution, when the skins were incubated at ambient temperature (28 ± 2°C). In the case of the undiluted crude enzyme, hair loosening started after 6 h of application, and at the end of the 8th h all the hair came away easily, whereas in the 2-fold-diluted crude enzyme, hair loosening started after the 8th h and came away only at the end of the 12th h. Although a much elaborate study is needed to standardize the proteolytic dehairing for leather processing per se, the results have demonstrated that the protease produced from a cheap substrate like fish meal has the ability to perform dehairing even at dilute concentrations.

Cost Comparison of Control and Cost-Effective Media

Table 4 summarizes the approximate costs of components used in the original multicomponent medium and fish meal media for production of 100 L of enzyme solution taken as a basis. The cost for 100 L is USD 34 with the standard medium, whereas it is only USD 2.4 for fish meal, indicating about a 15-fold cost benefit obtained mainly because of elimination of all the expensive components. Although the realistic cost-benefit calculation involves several other factors, if a reduction in the raw material cost is achieved then it is a major step towards reduction of the final product cost.

Fish meal is generally used as a food ingredient for fish, poultry, and animals, since it has high nutritional value. At the same time, it is also worthwhile to exploit its nutritional

![Fig. 9. Dehairing of goat skin: (A) raw skin, (B) dehairing with crude enzyme, and (C) dehairing with 2-fold-diluted crude enzyme.](image)
and cost-effective characteristics for protease production aiming for better economics. The handful of reports that have highlighted fish processing by-products to be a cheaper protein source than conventional ingredients (viz., peptone, yeast extract, etc.) for protease production, however, have not been tried with fish meal. In this paper, recognizing the presence of major nutrients, fish meal has been used in place of the costlier protein and carbon source for alkaline protease production from *Bacillus pumilus* MTCC 7514, and subsequently for application of the protease in de毛ing of goat skin. Looking at the biomass profiles as well as the activity, it is clear that fish meal by itself serves as a potential carbon and nitrogen source. The 15-fold cost reduction that is achieved by its substitution is quite significant. It is worth mentioning that an inherent compatibility pre-exists in the system, with both substrate and organism being marine resources. The scalability of protease production at the fermenter level, higher yields, cost benefit vi·s-á-vi·s the conventional medium, and the dehauling potential sans any concentration step renders this bioprocessing strategy commercially viable. The process can be improved substantially through optimization techniques examining all the factors influencing the standardization of the process at a pilot scale and semi-commercial scale, which is of course necessary to convert this into a viable technology. Moreover, the potential of the enzyme in other applications is required to be examined.

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