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Original Article

Solid state fermentation of fish feed with amylase producing bacteria

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Abstract
A formulated fish feed was fermented with amylase producing *Bacillus cereus* (Sn-1 and Sn-3) and Proteus mirabilisis -Sn-2 employing solid state fermentation (SSF) at their corresponding optimized growth conditions. SSF of the fish feed indicated significant increase and decreases in glucose and starch contents within seven days, respectively. Significant elevations also occurred in protein levels as compared to the corresponding values of non fermented control feed. The protein levels increased up to 42.27% by *B. cereus*-Sn-1 after 24 hours, 63.16% by *P. mirabilis*-Sn-2 after 168 hours and 47.47% by *B. cereus*-Sn-3 after 168 hours of incubations. These bacterial isolates caused the nutritional increments with 10% inocula and 70% moisture contents. The C.F.U./g of fermented feeds paralleled, in general, the feed nutritional enrichment. These results are reminiscent to design commercial level solid state fermentation facilities for developing the aquafeed industry in this country.
Key words: Fish feed, fermentation, biochemical profile.

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INTRODUCTION

Fish is a nutrient rich food containing high amount of protein with high biochemical value for humans. Feeds and feeding are among the most important aspects of day-to-day management of cultured fish species, and a proper understanding of this area is vital for health of the animals being cultured and thus profitability of commercial fish culturing (De Silva, 1999). Almost 40% of aquaculture production is now firmly dependent on commercial feed (Tacon, 2002). Thus improving feed efficiency, especially for the industrial systems is imperative (Naylor *et al*., 2000). Considering the importance of nutritionally balanced and cost-effective artificial diets for fish, there is an increasing research effort to evaluate the nutritive value of different non-conventional feed resources, including terrestrial and aquatic macrophytes (Mondal and Ray, 1999).

The Indian major carp, *Labeo (L) rohita* is primarily a herbivorous to omnivorous species and prefers to feed on plant materials (Talwar and Jhingran, 1991). Owing to its economic importance the fish species is being extensively cultured in Pakistan. Deciding a feed ingredient as a source of nutrients for assimilation by the animal is very important. Fish meal, owing to its nutritional quality, is an important component of feed for most cultivable fish species. Plant protein sources, which are more consistently available and cheaper to produce than fish meal/other animal proteins, have been extensively used in combination with fish meal in aquafeeds. Another way to enrich fish feed is through exploitation of microbial activity. For several centuries, fermented products derived from plant or animal materials have been an acceptable and essential part of diet in most parts of the world. Health benefits have also often been associated with them (Kalantzopoulos, 1997). Winsen *et al*. (2001) reported that fermented feed contains high concentrations of lactic acid, several volatile fatty acids (acetic acid, butyric acid, and propionic acid), large number of lactobacilli and has a low pH (Winsen *et al*., 2001). Processing plant materials through a simple and inexpensive method like fermentation might considerably decrease the antinutritional factors and crude fibre content thereby increasing their nutritional value (Tacon, 1993; Ray and Das, 1995; Mondal and Ray, 1999).

Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those

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processes where the crude fermented product may be used directly as the enzyme source. A large number of microorganisms including bacteria, yeast and fungi produce different groups of enzymes.

Selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. Besides its several other applications, SSF has also been employed to produce amylases (Ray *et al*., 1997). Although commercial production of amylases is carried out using both fungal and bacterial cultures, bacterial α-amylase is generally preferred for starch liquefaction due to its high temperature stability.

In the present study three amylolytic bacterial isolates were employed to saccharify and ferment a formulated fish feed. Provision of such fermented feed will likely enhance growth of culuted fish.

MATERIALS AND METHODS

Known amylase producing bacterial strains designated as Sn-1, Sn-2 and Sn-3, from the bacterial culture depository of Microbial Biotechnology Laboratory, Department of Zoology, University of the Punjab (Quaid-e-Azam campus), Lahore were employed for this study.

Optimization of the bacterial growth conditions

Bacterial strains were revived in nutrient broth and then optimized for temperature (30, 37 and 50 °C), oxygen (aerobic, anaerobic natures), pH (5, 7 and 9) and inoculum size (1, 5 and 10%). All these and subsequent experiments were performed in triplicates and 24 hours old cultures were used as inocula.

Characterization of bacterial isolates

The bacterial isolates were processed for routine Gram's, endospore, capsular and flagellar stainings and motility test. Fresh bacterial growths were also processed for oxidase, catalase, lecithinase, nitrate reduction, citrate utilization, indole, sulphide, obligate and facultative anaerobic nature determination bacterial, carbohydrate utilization and lactose utilization tests.

Nitrate reduction and carbohydrate utilization tests were proceeded according to Collins *et al.* (1995). Citrate utilization, sulphide, facultative anaerobic bacterial and lactose utilization tests were carried out following the methods given in Merck (1996-97). While the remaining tests were performed as described by Benson (1994). Based upon the above mentioned phenotypic characterization the bacterial isolates were identified.

SSF of formulated fish feed

For solid state fermentation, an apparatus was designed and installed according to Hofrichter *et al.* (1999). Twenty grams formulated fish feed (molasses, 4%; fish meal, 5%; rice polishing, 34.3%; ground nut oil cake, 53.7%; table salt, 1%; dicalcium phosphate 1% and vitamin premix, 1%) were taken in a glass container (12 cm length x 06 cm diameter) and autoclaved. The moisture content provided was 70% with 10% bacterial inocula. Each container was fitted with an inlet pipe which provided filtered aeration by an aerator. The bioreactors were also provided with aseptic outlet for exhaust and made safe from contamination by sealing the inlet/outlet joints as well as the brim of main opening with molten paraffin wax. The whole apparatus was incubated at an optimum temperature range of each bacterial isolate for 7 days, with a constant flow of filtered sterilized air. Samples were taken after 24, 48, 72, 120 and 168 hours under aseptic conditions. The fermentation material was stirred and distilled water added to replenish its 70% (v/w) moisture content on daily basis. Control jars were processed similarly but without the bacterial inoculation.

C.F.U./g of fermented fish feed

One gram fermented feed was sampled in 9 ml saline solution and then serially diluted. From each dilution 100 µl was spread over 20ml of solidified nutrient agar medium. After 24 hours, incubation at 37 °C, colonies were counted for the C.F.U. calculation.

Biochemical analysis of feed

Fresh feed (0.5g) was taken and homogenized in 4.5 ml saline solution following centrifugation at 5000 rpm at 4 °C for 10 minutes. The supernatant was processed for the estimation of total carbohydrate, glucose,

protein, starch and amylases according to the methods of Dubois *et al.* (1956); Hartel *et al.* (1969); Lowry *et al.* (1951) and Miller (1959) respectively. Samples of fermented feeds were processed similarly.

Statistical analysis

Statistical analysis was performed according to program SPSS 12. For comparison of two and more than two groups, Students'-t-test and single factor analysis of variance were applied, respectively.

RESULTS

The bacterial isolates were identified based upon their phenotypic characterization and presented in Table I.

Table I: Characterization of select bacterial isolates.

Parameters	Bacterial Isolate		
	Sn – 1	**Sn – 2**	**Sn – 3**
Gram staining	Gram +ve, diplobacilli	Gram –ve, rod shaped	Gram +ve, diplobacillus
Cell size	3.1µm	3.0 µm	3.0 µm
Endospore	+	+	+
Flagella	Unipolar, tuft of flagella	Unipolar, tuft of flagella	Unipolar, tuft of flagella
Motility	+	+	+
Capsule	-	-	-
Oxidase	+	-	+
Catalase	+	+	+
Nitrate reduction	-	+	+
Lecithinase	+	+	+
Sulphide reduction	+	+	-
Oxygen requirement	Facultative anaerobes	Facultative anaerobes	Facultative anaerobes
Carbohydrate utilization	+	+	+
Lactose utilization	N.A.	-	N.A.
Citrate utilization	N.A.	-	N.A.
Indole test	N.A.	-	N.A.
Identified as	*Bacillus cereus* – Sn-1	*Proteus mirabilus*- Sn-3	*Bacillus cereus* – Sn-1

N.A.: Not applied

Growth conditions optima revealed that the bacteria *Bacillus cereus* Sn-1 and *Proteus mirabilis* Sn-2 grew best at 37 °C while the bacterium *Bacillus cereus* Sn-3 showed highest growth when incubated at 30 °C. All the three bacteria showed best growth at initial pH 5 and at 10% inoculum. Regarding the oxygen requirement the three bacterial species appeared facultative anaerobes (Table II).

The bacterium *Bacillus cereus* Sn-1 expressed significant increase of glucose contents in the fermented feed. The values turned out about 44%, 20% and 58% higher after 24, 72 and 168 hours of fermentation, respectively than the control value (Table III). While feed starch content decreased significantly down to 17%, 23%, 40% and 28% after 48, 72, 120 and 168 hours of the fermentation, respectively.

Table II: Optimization of bacterial growth conditions after 24 hours of incubation

Parameters		Sn-1	Sn-2	Sn-3
Temperature	30°C	0.07^{a}± 0.003	0.17^{a}± 0.009	0.17± 0.027
	37 °C	0.24^{ab}± 0.023	0.17^{a}± 0.004	0.12± 0.008
	50 °C	0.04^{b}± 0.015	0.11^{b}± 0.007	0.14± 0.059
pH	5	0.67^{a}± 0.032	0.41± 0.059	0.72^{a}± 0.046
	7	0.27^{b}± 0.051	0.27± 0.017	0.43^{b}± 0.042
	9	0.40^{ab}± 0.042	0.36± 0.025	0.49^{b}± 0.053
Inoculum size	1%	0.76^{a}± 0.014	0.29^{a}± 0.005	0.79± 0.003
	5%	0.84^{ab}± 0.024	0.32^{a}± 0.034	0.79± 0.042
	10%	0.92^{a}± 0.033	0.56^{b}± 0.021	0.89± 0.053
Oxygen requirement	+ve	0.19± 0.017	0.50± 0.027	0.57± 0.029
	-ve	0.18± 0.010	0.52± 0.025	0.52± 0.013

Value represent A_{600nm} and are Mean ± S.E.M. of triplicates. The values without a common alphabet are significantly different from each other in the respective columns. Student t-test and analysis of single factor variance are at $p \leq 0.05$.

Table III: Effects of SSF on total carbohydrates and glucose contents of fish feed.

Incubation hours	Total carbohydrates (mg/g)			Glucose contents (mg/g)		
	Sn-1	Sn-2	Sn-3	Sn-1	Sn-2	Sn-3
24	21.80^{a}±0.19 (20.85±0.31)	14.66±1.19 (20.85±0.31)	12.55^{ab}±2.29 (20.85±0.31)	7.10^{a}±0.50 (4.94±0.12)	13.66^{a}±1.92 (4.94±0.12)	7.13±0.88 (4.94±0.12)
48	19.82^{ab}±1.31 (20.52±0.19)	15.72±1.41 (20.52±0.19)	17.89^{a}±1.34 (20.52±0.19)	4.45^{ab}±0.74 (5.52±0.08)	3.08^{b}±0.94 (5.52±0.08)	5.48±0.11 (5.52±0.08)
72	22.45^{a}±0.27 (20.63±0.29)	14.58±2.68 (20.63±0.29)	12.89^{ab}±0.16 (20.63±0.29)	5.31^{ab}±0.78 (4.42±0.16)	6.14^{b}±1.30 (4.42±0.16)	4.60±1.31 (4.42±0.16)
120	15.70^{bc}±2.03 (20.72±0.28)	14.51±1.95 (20.72±0.28)	9.92^{b}±1.48 (20.72±0.28)	3.89^{b}±0.23 (4.85±0.13)	6.83^{b}±0.12 (4.85±0.13)	4.8±0.95 (4.85±0.13)
168	18.89^{ac}±0.95 (20.66±0.84)	19.56±1.63 (20.66±0.84)	7.84^{b}±0.87 (20.66±0.84)	7.12^{a}±0.79 (4.66±0.06)	15.05^{a}±1.15 (4.66±0.06)	5.16±0.87 (4.66±0.06)

Values are Mean ± SEM of triplicates. Control feed values are given in brackets. The values not sharing a common alphabet are significantly different from each other in the respective rows. Analysis of single factor variance is at $p \leq 0.05$.

Table IV: Effects of SSF on starch contents and α-amylase of fish feed.

Incubation time (hours)	Starch contents (mg/g)			α-amylase (mM/g)		
	Sn-1	Sn-2	Sn-3	Sn-1	Sn-2	Sn-3
24	0.73^{a}±0.01 (0.76±0.03)	0.63±0.04 (0.76±0.03)	0.16±0.02 (0.76±0.03)	0.09±0.007 (0.06±0.001)	0.06±0.033 (0.05±0.001)	0.02±0.004 (0.05±0.001)
48	0.63^{b}±0.09 (0.76±0.02)	0.37±0.09 (0.76±0.02)	0.35±0.11 (0.76±0.02)	0.06±0.015 (0.06±0.001)	0.03±0.013 (0.05±0.001)	0.10±0.047 (0.05±0.001)
72	0.57^{ab}±0.07 (0.75±0.06)	0.38±0.03 (0.74±0.06)	0.51±0.09 (0.75±0.06)	0.04±0.005 (0.06±0.001)	0.01±0.003 (0.05±0.002)	0.01±0.003 (0.05±0.001)
120	0.45^{b}±0.03 (0.75±0.01)	0.37±0.14 (0.74±0.004)	0.24±0.07 (0.74±0.004)	0.05±0.016 (0.06±0.001)	0.05±0.012 (0.05±0.002)	0.08±0.031 (0.05±0.002)
168	0.54^{ab}±0.06 (0.75±0.02)	0.37±0.09 (0.75±0.02)	0.52±0.08 (0.75±0.02)	0.06±0.015 (0.06±0.001)	0.06±0.009 (0.05±0.002)	0.03±0.010 (0.05±0.002)

For details of statistical analysis, see Table II.

Table V: Effects of SSF on total protein and C.F.U. of fish feed.

Incubation time (hours)	Total Proteins (mg/g)			C.F.U./g		
	Sn-1	Sn-2	Sn-3	Sn-1	Sn-2	Sn-3
24	17.59^{a}±0.16 (12.36±0.18)	18.75^{a}±0.21 (12.36±0.18)	13.01^{a}±0.50 (12.36±0.18)	3×10^{2}	154.27×10^{9}	12.941×10^{9}
48	15.24^{bc}±0.68 (12.32±0.08)	11.49^{b}±1.31 (12.32±0.08)	13.09^{a}±8.92 (12.32±0.08)	3.9×10^{5}	3.41×10^{10}	3.12×10^{10}
72	15.21^{bd}±0.28 (12.33±0.02)	11.63^{b}±0.25 (12.33±0.02)	12.68^{a}±1.02 (12.33±0.02)	4.35×10^{7}	73.84×10^{10}	12.1755×10^{11}
120	14.76^{b}±8.52 (12.48±0.02)	17.12^{a}±0.98 (12.48±0.02)	17.73^{b}±0.70 (12.48±0.02)	$3.7.58 \times 10^{9}$	TNC	51.15×10^{13}
168	16.67^{acd}±0.20 (12.51±0.02)	20.41^{a}±0.23 (12.50±0.02)	18.45^{b}±0.60 (12.51±0.02)	2.02445×10^{10}	TNC	TNC

For details of statistical analysis, see Table II; TNC=Too many to count

The α-amylase showed significant rise of 43.04% after 24 hours of incubation (Table IV). Total carbohydrate could increase only up to 4.52% and 8.81% after 24 and 72 hours post incubation while the parameter showed significant decrease of 24.23% after 120 hours of incubation (Table III). Protein level also showed rise (Table V). The bacterium *Proteus mirabilis* Sn-2 caused highly significant increases in glucose content upto 177% and 223% after 24 and 168 hours, respectively. While starch levels showed continuous

decreases during fermentation and the losses approached 17%, 52%, 50%, 51% and 51% at 24, 48, 72, 120 and 168 hrs. of fermentation, respectively as compared to control (Table IV). The α-amylase fluctuated non significantly during the fermentation process. While protein showed significant elevations at termination of the fermentation as compared to 2nd and 3rd sampling points (Table V).

Bacillus cereus Sn-3 did show increases in glucose levels up to 44% and 11% at 24 and 168 hours post incubation, respectively. While starch content decreased significantly down to 79% and 54% after 24 and 48 hours of incubation, respectively as compared to control (Tables III-IV). Total carbohydrate level consistently fell down throughout the fermentation, except at second observation. The α-amylase levels increased at 48 and 120 hours post incubation up to 66% and 30%, respectively (Table IV). At termination of the experiment highest protein content were found for the feed fermented by *P. mirabilis* – Sn2 (Table, VI).Total protein contents showed progressive rise till day seven of fermentation (Table V). C.F.U./g of bacterial strains showed that all isolates increase in number till 120 hours after incubation. After 168 hours post incubation *Bacillus cereus* Sn1 showed decline in number, while the other two bacterial strains continued to increase in number (Table V).

DISCUSSION

Solid-state fermentation (SSF) has useful applications in fermented feeds. Animal feed industry has been utilizing several products of microbial fermentations as feed additives with appealing growth promoting efforts (Saucedo *et al*., 1992; Macey and Coyne, 2005). This study reports improvement of nutritive values of a formulated fish feed fermented with locally isolated bacteria. Some important nutritional contents of the fermented fish feed increased in the range of 20-100% within seven days of the incubation.

Ability of certain fish species to hydrolyze (digest) complex carbohydrates is limited due to weak amylolytic activity in their digestive tracts. Thus for such species, starch digestion decreases as the proportion of dietary starch is increased (Hasan, 2000; Chen *et al*., 2006). On the other hand, starchy represents low cost and easily available feed ingredient. Thus percentage of a starch source is desired to be increased in fish feed. In this context the bacterial isolates employed in the present study appear efficient for converting starchy contents of fish feed into easily assimable glucose monomers.

The bacterial isolate *P. mirabilus* Sn2 was found to produce smaller (0.063 mM/g) amount of amylase. While the bacteria *B. cereus*-Sn1 and *B. cereus*-Sn3 could yielded up to 0.090 and 0.103 mM/g of the enzyme, respectively. Microorganisms are well known for their abilities to produce a variety of enzymes such as amylases, polysaccharides, cellulases, proteases, lipases and lignocellulases (Iyayi and Losel, 2001; Nguyen *et al*., 2007). These enzymes convert the polymeric forms of the macromolecules to soluble monomers. Bioconversion of starch and sugars to proteins including single cell protein is promising for upgrading animal feeds (Manilal *et al*., 1985).

Total carbohydrate content of the fish feed after fermentation did not show any significant increase. Rather there was found a declining trend attributed to bacterial consumption. Glucose contents achieved up to 177% (13.66 mg/g) and 223% (15.05 mg/g) elevations after 24 and 168 hours of incubation, respectively with *Proteus mirabilis*-Sn-2.. The *B. cereus* isolates also increased glucose up to 44% (7.13 mg/g) and 53% (7.12 mg/g) after 24 and 168 hours of fermentation, respectively. Increase in soluble sugars has also been reported by Iyayi and Aderolu (2004) in agro industrial byproducts with *Trichoderma viride*. Excessive monosaccharides liberation is traced to strong enzyme activation (Habijanic and Berovic, 2000). Starch contents showed a constant decreasing trend throughout the study period. Bacterial isolates Sn-1, Sn-2 and Sn-3 showed 40%, 52% and 79% decreases in the parameter at 120, 48 and 24 hours of fermentation, respectively.

It is well established that protein rich fish feed is more growth promoting (Kaushik, 2000; Refstei *et al.*, 2001). Significantly several folds increases of protein contents of SSF feed in this investigation over the control values are highly appealing to speculate the growth promoting

effects on the feed fed fish. *B. cereus* (Sn-3) and *P. mirabilis* (Sn-2) enhanced total protein contents up to 48% (18.45 mg/g) and 63% (20.41 mg/g), respectively after 168 hours of fermentation. Comparable results have been reported by Raimbault *et al*. (1985) when they cultured cassava peels with *Aspergillus sp*. The authors reported several fold increase in the protein contents. Such protein enrichment has been reported for barley, wheat, cassava and dehydrated beet pulp following their SSF (Duru and Uma, 2003).

The results of this study have shown that bacterial solid state fermentation is effective for enhancing the nutritive value of formulated fish feed through starch saccharification and single cell associated protein enrichment. This *in vitro* investigation provides baseline for practical applications of solid state fermentation in improving nutritional status of fish feeds.

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