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Effect of solid state fermentation on nutrient composition of selected feed ingredients

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ABSTRACT

Solid-state cultivation of the fungus *Aspergillus niger* and the bacterium *Bacillus coagulans* was carried out to enrich the nutritional value of plant ingredients like soybean meal, mixed ingredients and wheat bran to use as aquafeed ingredients. Fermentation of soybean meal (FSBM) using *B. coagulans* for 48 h resulted in significant ($p<0.05$) increase in the crude protein content (@ 3 to 7%) with concurrent decrease in nitrogen free extract (NFE) (11 to 16 %). Among the essential amino acids, valine (7%), isoleucine (2%), leucine (2%), lysine (93%) and tryptophan (42%) showed substantial increases in FSBM after 48 h. Solid state fermentation (SSF) of ingredient mix using *A. niger* NCIM 616 resulted in initial reduction of crude protein content during the first 48 h followed by significant ($p<0.05$) increase of 4 to 14% during the course of fermentation. The crude fat content showed a 35% increase in 96 h. Nitrogen free extract though increased marginally (4%) at 48 h showed significant reduction (17%) at 96 h. A marginal increase in arginine, valine and methionine levels were also observed in the fermented ingredient mix (FIM). Solid state fermentation of wheat bran using *A. niger* $S_1$4 (a mangrove isolate) had resulted in substantial increase in crude protein level (57 to 66%) as compared to that of raw wheat bran. The carbohydrate content in wheat bran showed substantial reduction (75 to 39%) during the course of fermentation. Essential amino acids like, histidine, threonine, valine, isoleucine and lysine showed increase during SSF. The results of the present study show that *B.* coagulans and the selected strains of *A. niger* can be used for nutritional enrichment of plant ingredients for further use in aquafeed formulations.

Introduction

Aquaculture is one of the fastest growing food-producing sectors, providing an acceptable supplement to and substitute for wild fish and plants (FAO, 1997). To sustain a high rate of growth, a matching increase in feed production is imperative (Francis *et al*., 2001). The production of aquafeeds is expected to rise from the current level of about 13 million metric tonnes (mt) to about 30 mt in 2010 and it is estimated that a minimum of 3 mt of fishmeal equivalent, alternative protein sources will be required in the aquaculture industry annually by the year 2010 (Francis *et al*., 2002). In order to attain a more economically sustainable, eco-friendly and viable aquaculture production, research interest has been directed towards the evaluation and use of unconventional protein sources, particularly from plant products such as seeds, leaves and other agricultural by-products (El-Sayed, 1999; Siddhuraju and Becker, 2001). The main limitations of plant proteins are the deficiencies in certain essential amino acids and minerals, and the presence of anti-nutritional factors and complex carbohydrates (NRC, 1993; Vielma *et al*., 2004). Therefore, in searching for cheaper raw materials to be used in feed production, it is crucial to obtain higher nutritional values than traditional substrates, in order to minimize the need for these high cost additives, and thereby maintain the economic viability of animal production (Villas-Boˆas *et al*., 2002). Utilization of non-conventional resources for animal feed has been made possible by the process known as solid-state fermentation (SSF) (Pandey *et al.*, 2000 and 2001). Microorganisms can be cultivated on agro-industrial products with production of large amounts of cells rich in proteins that commonly contain all the essential amino acids, in addition to favorably high vitamin and mineral levels (Kuhad *et al*., 1997). Furthermore, new applications of SSF have been suggested for the production of antibiotics (Barrios-Gonzales *et al*., 1988), secondary metabolites (Trejo-Hernandez *et al*., 1993) or enriched food stuffs (Senez *et al*., 1980). The process of SSF has been reported to upgrade the nutritional quality of agro industrial products that can be used in aquafeed and animal feed industries (Singh *et al.*, 1990; Lena *et al*., 1997, Vijayakumar, 2003, Imelda-Joseph and Paulraj, 2003). The objectives of the present study were to determine the nutritional profile of soybean meal, mixed ingredients, and wheat bran in SSF using bacterial strain *Bacillus coagulans* and fungal strains *Aspergillus niger* NCIM 616 and *Aspergillus niger* $S_1$4 at different durations.

Materials and methods

Microorganisms

Aspergillus niger NCIM 616 was obtained from the National Collection of Industrial Microbes (NCIM), Pune, India and *Aspergillus niger* $S_1$4 was isolated from a local mangrove swamp. The bacterial strain *Bacillus coagulans* was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

Substrate and the process of SSF

Soybean meal, mixed ingredients (soybean meal, wheat flour, groundnut oil cake and sesame oil cake in the ratio 4:3:2:1 and wheat bran were purchased from the local market and used as substrates for SSF.

Three sets of experiments were conducted to evaluate the changes in the nutrient profile of different ingredients by SSF. In experiment I, 50 g soybean meal with 60% moisture was inoculated with 18 h old *B. coagulans* (1% inoculum of 10^7 to 10^8 cells ml^{-1}) and incubated at 30 ±1°C and pH of 6.5 to 7.0 for 12, 24, 36 and 48 h.

In experiment II, 50 g mixed ingredients with 60% moisture fortified with mineral mixture (KH_2PO_4- 0.0035%; $MgSO_4$. $7H_2O$ - 0.5%; $MnSO_4$. $7H_2O$ – 0.0028%; $FeSO_4$. $7H_2O$ – 0.0087%; $ZnSO_4$. $7H_2O$ – 0.0025% and $CaCl_2$ – 0.0035%) were inoculated with *A. niger* NCIM 616 (@ 10 ml of 10^6 spores ml^{-1} in 0.1% Tween- 80) and incubated at 30°C for 48, 72, 96 and 120 h.

In experiment III, wheat bran with 60% moisture was fortified with czapek dox [$NaNO_3$ (2.5 g l^{-1}), K_2HPO_4 (1 g l^{-1}), $MgSO_4$. $7H_2O$ (0.5 g l^{-1}), KCl (0.5 g l^{-1}) pH @5.0] (Aikat and Bhattacharya, 2000), and inoculated with *A. niger* $S_1$4 (@ 10 ml of 2 x 10^6 spores ml^{-1}), and incubated at 30°C for 8 days with sampling at every 24 h. For inoculum, 7 days old slants of *A. niger* strains maintained in potato dextrose agar (PDA, Himedia, Mumbai) were used and sterile Tween-80 (0.1%) was added to make the spore suspension and the initial pH was 6.4-6.5.

All the three experiments were conducted with three replicates for each treatment in 500 ml conical flasks (Borosil, India) and the flasks were kept under stationary condition with occasional shaking.

Chemical analysis

For experiment I, after every 12 h and for experiments II and III, after every 24 h, the fermented products were dried to a constant weight, ground to <1mm size and proximate composition analyses were carried out. Prior to the start of the three experiments, initial proximate analyses were done for all the substrates. Chemical analysis of unfermented and fermented samples included moisture content, crude protein, crude ash, crude fat and nitrogen free extract (NFE) (AOAC, 1990). Amino acids profile was determined using HPLC (Waters India Ltd.) after acid hydrolysis. Tryptophan was determined spectrophotometrically after alkali hydrolysis (AOAC, 1990). The results were analyzed by two-way ANOVA.

Results and discussion

The raw materials used for SSF of ingredient mix had been mixed in the proportion 4:3:2:1, soybean flour: wheat flour: groundnut oil cake: sesame oil cake, respectively, to obtain a final mix with 35-40 % protein, which is the recommended protein level for *Penaeus monodon* post-larvae (Alava and Lim, 1983; Akiyama *et al*., 1992). Wheat bran was selected for the present study because it is a major agro-industrial by-product widely used as a supplement in cattle feed and fish feed (Mitra *et al.*, 1996; Lena *et al.*, 1997; Aikat and Bhattacharya, 2000).

During SSF, mild colour change was observed for the raw materials (light brown for FSBM and black for FIM and FWB). The initial neutral pH of the substrate (6.5 to 7.0) got reduced to acidic (4.5- 5.0) during the course of fermentation of all the substrates. The reduction in pH may be due to the organic acid production by the microorganisms and release of carbon-di-oxide. Alexander (1961) reported that during the fermentation process, substrate pH falls to the range of 5.0 to 5.5 initially and that modification of substrate pH in fermentation process is greatly-influenced by the release of ammonia (if basic) and carbon-di-oxide (if acidic).

The proximate composition of the raw materials (soybean meal, wheat flour, groundnut oil cake, sesame oil cake and wheat bran) used for fermentation as substrates are given in Table 1. Solid state fermentation of soybean

Table 1. Proximate composition of raw materials used for fermentation (dry matter basis)

Raw material	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Crude ash (%)	Nitrogen free extract (%)
Wheat flour	16.79±0.03	1.53±0.41	0.37±0.17	1.1±0.41	80.21±2.41
Soybean meal	45.97±0.21	0.81±0.02	0.82±0.08	6.89±0.01	45.41±0.08
Groundnut oil cake	33.17±2.12	5.2±0.71	4.97±0.12	17.27±0.17	39.39±0.89
Sesame oil cake	45.84±0.18	6.94±0.48	2.21±0.06	10.85±0.94	34.16±2.79
Wheat bran	17.03±0.06	4.96±0.06	**	3.77±0.02	73.89±0.61*

* Total Carbohydrate; ** Not determined

Table 2. Proximate composition of soybean meal after fermentation with *Bacillus coagulans* (% dry matter)

Time (h)	Moisture (%)	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Crude ash (%)	Nitrogen free Extract (%)
Control	4.80±0.15	45.97±0.21	0.81±0.02	0.82±0.08	6.89±0.01	45.41±0.08
12	3.82±0. 14	47.21±0.15	0.34±0.05	3.41±0.10	8.65±0.02	40.26±0.18
24	4.97±0.05	47.47±0.23	0.50±0.03	2.89±0.06	8.65±0.01	40.19±0.14
36	6.57±0.11	48.63±0.16	0.70±0.04	2.68±0.08	8.17±0.02	39.60±0.21
48	6.82±0.13	49.32±0.11	0.70±0.08	2.56±0.06	8.36±0.01	38.25±0.80

meal, ingredient mix and wheat bran using *B. coagulans*, *A. niger* NCIM 616 and *A. niger* $S_1$4 respectively, had resulted in considerable variation in the nutritional profile.

The proximate composition as well as amino acids profile of FSBM and control, FIM and control and FWB and control are given in tables 2 to 7. The crude protein content increased with duration of fermentation for all the three substrates with the maximum at 48h (7%) for FSBM, 96 h (14%) for FIM and on day 8 (49%) for FWB. The significant ($p<0.05$) increase in protein content during fermentation may be attributed to the efficient bioconversion of highly polymerized carbohydrates into microbial protein and the production of different types of enzymes, which are proteinaceous in nature (Vijayakumar, 2003; Bhatnagar, 2004). Mitra *et al*. (1996) have reported that by the process of SSF it was possible to convert cassava to a protein enriched animal feed and the highest increase in protein content observed was 14.32% from the initial 1.28% by filamentous fungi. *A. niger* is reported to produce as many as 19 enzymes (Pandey *et al*., 1999) which are proteinaceous in nature. The initial decrease in crude protein content in FIM indicates that *A. niger* utilize the available nitrogen for its vegetative growth initially, followed by the synthesis of protein through the process of bioconversion resulting in an increase in protein content of substrate with extended duration of fermentation. Similar observations in protein enrichment were reported by Singh *et al*. (1990) and Arora *et al*. (2000) for fermented potato process waste using *Rhizopus oryzae*. Lena *et al.* (1997) have also reported increase in crude protein content of wheat bran during SSF with white-rot fungus.

Table 3. Amino acid profile of soybean meal before and after fermentation with *B. coagulans* (100 g protein^{-1})

Amino acids	Control	12 h	24 h	36 h	48 h
Aspartic acid	6.99	9.33	8.16	9.59	9.48
Glutamic acid	11.93	14.10	13.02	14.19	14.34
Serine	7.42	6.50	6.96	6.28	6.30
Glycine	11.14	7.70	9.42	7.53	7.39
Histidine	3.00	2.55	2.78	2.56	2.49
Arginine	5.67	5.02	5.35	5.13	5.31
Threonine	4.81	4.14	4.48	4.11	4.13
Alanine	5.27	6.01	5.64	6.26	6.20
Proline	8.94	6.89	7.92	6.75	6.65
Tyrosine	3.22	2.69	2.96	2.76	2.68
Valine	4.33	4.46	4.39	4.69	4.64
Methionine	1.11	1.11	1.11	1.09	0.86
Cystine	0.94	0.60	0.77	0.71	0.55
Isoleucine	4.00	3.99	4.00	4.13	4.07
Leucine	8.04	8.14	8.09	8.25	8.20
Phenylalanine	5.99	5.08	5.53	4.62	4.59
Lysine	5.44	9.81	7.62	10.36	10.54
Tryptophan	1.65	2.50	2.07	2.51	2.34

Among the essential amino acids, valine (7%), isoleucine (2%), leucine (2%), lysine (93%) and tryptophan (42%) showed considerable increase in FSBM after 48 h (Table 3). Marginal increase in essential amino acids like valine, arginine and phenylalanine was observed during SSF of FIM (Table 5). The amino acid profile of control as well as the FWB is shown in Table 7. Significant increases in aspartic acid (43.71%), serine (69.79%), histidine (6.93%), threonine (70.57%), alanine (36.69%), valine (16.8%), cystine (40%) and lysine (43.77%) were observed on day 5. The level of amino acids in cellobiases, the enzymes produced by certain strains of *A. niger* showed high contents of aspartic acid, glutamic acid, threonine, serine, and glycine (Abdel-Naby *et al*., 1999). Single cell protein produced by *A. niger* contained 30.4% crude protein and had an essential amino acid profile featuring a high lysine content and appreciable amounts of methionine and tryptophan, and 12.9% fat, which comprised of all the essential fatty acids (Singh *et al.*, 1990). The increase in amino acids in the fermented product shows that the carbohydrate utilization is closely proportional to protein production during solid substrate fermentation. The reduction in certain amino acids like arginine may be due to the utilization of it for growth and production of enzymes and other organic compounds by the microorganisms during SSF.

The crude fat of FSBM showed a decrease ranging from 13.5 to 28% during the course of fermentation with the maximum reduction at 24 h (Table 4). The initial reduction may be due to the utilization of available fat for bacterial (*B. coagulans*) growth. The crude fat content (2.52%) in FIM derived by fermentation with *A. niger* NCIM 616 showed a decrease initially and showed 35% increase from the initial at 96 h, and afterwards showed a decreasing trend with extended duration of fermentation. It clearly suggests that initially *A. niger* NCIM 616 utilized

Table 4. Proximate composition of unfermented ingredient mix (control) and FIM on SSF using *A. niger* NCIM 616 (on dry matter basis)

Time (h)	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Crude ash (%)	Nitrogen free extract (%)
Control	35.04±0.12	2.52±0.31	2.65±0.19	7.63±0.14	52.16±0.56
48	31.02±0.44	2.33±0.06	4.19±0.19	8.45±0.21	54.01±0.52
72	36.29±0.82	3.09±0.41	3.65±0.19	9.43±0.15	47.54±0.78
96	39.95±0.38	3.41±0.39	3.56±0.53	9.90±0.08	43.18±0.62
120	38.91±0.68	2.95±0.17	3.48±0.47	10.70±0.17	43.96±1.41

Table 5. Amino acid percentage in the unfermented ingredient mix (control) and FIM

Amino acids	Control	24 h	48 h	72 h	96 h	120 h
Aspartic acid	9.55	9.39	9.33	9.33	9.65	8.69
Glutamic acid	15.82	15.66	15.98	16.17	15.96	15.91
Serine	6.66	5.16	5.58	6.08	6.50	4.80
Glycine	7.50	7.68	7.93	8.13	8.27	7.79
Histidine	2.28	2.44	2.31	2.26	2.22	2.41
Arginine	5.50	4.65	5.03	5.31	5.54	4.34
Threonine	4.12	3.44	3.59	3.85	3.93	3.18
Alanine	6.48	7.51	7.09	6.58	6.34	8.74
Proline	7.08	7.53	7.70	7.55	7.13	7.80
Tyrosine	2.46	3.35	2.97	2.70	2.50	3.45
Valine	4.56	6.42	5.97	5.34	4.57	6.95
Methionine	1.25	1.41	1.20	1.18	1.22	1.35
Cystine	0.6	0.6	0.48	0.59	0.58	0.38
Isoleucine	3.72	3.73	3.80	3.73	3.63	3.75
Leucine	7.85	6.79	7.19	7.49	7.77	6.74
Phenylalanine	4.35	4.74	4.56	4.42	4.36	4.96
Lysine	7.66	7.33	7.12	6.99	7.28	6.36
Tryptophan	2.56	2.50	2.07	2.52	2.34	2.51

Table 6. Proximate composition of nutrients of unfermented wheat bran (control) and FWB on SSF

Days	Crude protein	Crude fat	Crude ash	Total CHO*
Control	17.60±0.12	1.74±0.03	5.03±0.01	75.63±0.32
1	18.38±0.10	2.18±0.01	5.27±0.02	74.17±0.29
2	19.36±0.12	2.85±0.03	5.90±0.01	71.89±0.36
3	20.94±0.05	3.09±0.03	6.91±0.01	69.15±0.31
4	24.61±0.06	2.75±0.05	7.54±0.03	65.10±0.31
5	24.76±0.08	1.69±0.06	8.21±0.16	65.34±0.6
6	27.62±0.07	1.46±0.03	8.08±0.23	62.84±0.38
7	26.39±0.09	1.01±0.13	8.17±0.01	64.43±0.38
8	26.25±0.09	1.03±0.03	8.22±0.02	64.50±0.62

* Total Carbohydrate

protein and fat along with carbohydrates for their growth and subsequently, converted carbohydrates of the substrate to proteins and organic and fatty acids. After 96 h, the fat content of substrate got reduced, which may be due to the activity of the extracellular lipolytic enzymes produced by the fungus on the lipid and the fatty acids present in the substrate. Using *A. niger* $S_1$4, a similar trend was observed for wheat bran. The crude fat content showed an increase of about 78 % on day 3 and 58% on day 4 followed by a decrease of 48% on day 8 compared to that of the initial. The increase in crude fat content in the fermented wheat bran during SSF till day 4 may be attributed to the production of fungal fatty acids during fermentation. Fungi are reported to produce fatty acids at varying levels during SSF (Higashiyama *et al*., 2002). Kamini *et al*. (1997) reported that lipase activity of *A. niger* vary according to the strain and substrate used in the fermentation process.

Crude ash level in FSBM increased to about 17% in 48 h and that in FIM the increase was 10 to 40% in 120 h from the initial. For wheat bran, the increase ranged between 4 and 63%. The increase observed in crude ash may be due to the dry matter loss during fermentation causing a relative increase in the unaltered components of the fermented product, especially the fibre and ash contents. The dry matter loss was 20 % at 96 h of fermentation in FIM and for FWB on day 8, the loss was 38.36% from that of the initial.

Increase in the crude fibre content was observed in both FSBM as well as FIM. For FIM, the increase was 58% from the initial after 48 h of fermentation. It may be attributed to the utilization of easily digestible soluble carbohydrates by the growing fungus, leaving the indigestible fibre content high as reported by Singh *et al.* (1990).

Reduction in nitrogen free extract (NFE) was observed for FSBM as well as FIM during the course of fermentation. The maximum reduction was observed after 96 h (17%) for FIM. The results show that the available carbohydrate (NFE) of the substrate decreased with the increase in protein and fat contents of the substrate during SSF suggesting bioconversion of carbohydrates in the substrate into microbial protein and other compounds. For wheat bran, the total carbohydrate content was determined by indirect estimation by subtracting the total values of crude fat, crude protein and crude ash from 100 (Table 6). The total carbohydrate content showed a steady and significant ($p<0.05$) decrease during the fermentation possibly due to the breakdown of carbohydrate by the action of fungal amylases, releasing the simple and utilizable carbohydrate molecules for its metabolic activities. The reduction of total carbohydrates from 80.46% in the control to 63.05% (*i.e.*, @ 21.64% reduction) on day 8 in the fermentation

Table 7. Amino acid percentage in the unfermented wheat bran (control) and FWB

Amino acids	Control	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
Aspartic acid	7.07	7.1	8.01	9.26	9.29	10.16	10.09	8.89	8.85
Glutamic acid	16.84	17.37	15.27	10.45	12.26	9.44	9.44	9.51	9.28
Serine	5.43	5.93	7.04	7.64	8.23	9.22	9.07	7.98	7.25
Glycine	11.31	11.3	11.29	11.88	11.26	10.65	10.82	11.29	11.00
Histidine	2.31	2.45	2.58	2.41	2.41	2.47	2.46	2.29	2.50
Arginine	4.34	4.33	4.02	3.98	3.89	3.69	3.69	3.22	3.75
Threonine	3.50	3.90	4.55	5.21	5.33	5.97	5.86	6.40	5.32
Alanine	7.25	7.37	7.76	9.27	8.74	9.17	9.16	9.91	9.10
Proline	11.76	10.38	9.81	7.22	7.49	6.21	6.05	7.08	7.09
Tyrosine	2.43	2.39	2.39	2.66	2.49	2.44	2.41	2.41	2.61
Valine	4.94	5.38	5.69	6.21	5.94	5.77	5.82	5.67	5.99
Methionine	0.34	0.18	0.11	0.11	0.10	0.12	0.11	0.11	0.05
Cystine	0.15	0.21	0.24	0.21	0.2	0.21	0.24	0.14	0.12
Isoleucine	3.23	3.40	3.67	4.24	3.93	3.80	3.92	3.91	3.86
Leucine	7.41	7.34	7.05	7.76	7.44	7.22	7.42	7.87	7.52
Phenylalanine	3.99	3.85	3.53	3.66	3.51	3.23	3.38	3.54	3.42
Lysine	5.14	4.55	3.93	6.25	5.62	7.2	7.39	6.73	8.46
Tryptophan	2.94	2.25	1.83	2.16	1.14	1.75	1.94	1.60	1.78

process shows the continuous utilization of carbohydrates for the metabolic activities of *A. niger*.

The results of the present study suggest that the microbes *B. coagulans* and the selected *A. niger* strains are efficient to convert complex carbohydrates to simpler molecules with enrichment of microbial protein, and that the fermented ingredients are good sources of nutrients towards aquafeed formulations.

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