

Bio-processing of Linseed Oil-Cake through Solid State Fermentation by Non-Starch Polysaccharide Degrading Fish Gut Bacteria

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Abstract

Cellulose and hemicellulose (xylan) are the most common Non-Starch Polysaccharides (NSPs) present in plant ingredients that exhibit anti-nutritional effect. Degradation of cellulose and xylan has been investigated under solid-state fermentation (SSF) using oil-cakes, viz., groundnut oil-cake (GOC), mustard oil-cake (MOC), sunflower oil-cake (SOC), sesame oil-cake (SeOC) and linseed oil-cake (LOC) as substrates. Finally LOC was considered in the present study as it contained maximum amount of cellulose ($17.51 \pm 0.87 \text{ g } 100 \text{ g}^{-1}$) and xylan ($13.02 \pm 0.66 \text{ g } 100 \text{ g}^{-1}$). Bio-processing of LOC was carried out through SSF in two different combinations utilizing efficient cellulase- and xylanase-producing strains, *Bacillus pumilus* KF640221 (Set-I) and *Bacillus tequilensis* KF640219 (Set-II), isolated previously from the proximal intestines of rohu, *Labeo rohita* and silver carp, *Hypophthalmichthys molitrix*, respectively. Analysis of the fermentation sets revealed that *B. pumilus* KF640221 in Set-I was most effective in reducing the cellulose and xylan contents of LOC. Besides, SSF under optimized conditions caused considerable increase in crude protein, crude lipid, total free amino acids and total free fatty acids along with reduction in the contents of other anti-nutritional factors, e.g., crude fiber, tannins, phytic acid and trypsin inhibitor. High performance liquid chromatography (HPLC) analysis of raw and fermented LOC of Set-I indicated that concentrations of methionine, histidine, tryptophan, phenylalanine, threonine were increased considerably. Further research is inevitable to explore the possibilities for utilization of SSF-processed LOC to set up a strategy for sustainable utilization of low cost oil-cakes as animal feed ingredients.

Keywords: Cellulose; Xylan; Bacillus; Solid state fermentation; Linseed oil-cake

Introduction

Oil-cakes are the by-products obtained after oil extraction from the seeds. Being rich in protein, oil-cakes are frequently can be used as animal feed ingredients, especially for ruminants and fish [1]. However, beside deficiencies in some of the essential amino acids, the direct incorporation of oil-cakes in animal feed has been restrained by the presence of antinutritional factors (ANFs), majority of which are polyphenols, trypsin inhibitors, phytic acid and Non-Starch Polysaccharides (NSPs) [2]. NSPs comprise up to 90% of the plant cell wall [3], wherein cellulose, hemicellulose and pectin are the most abundant [4]. Xylan is considered as the major structural component of plant cell wall and the most abundant copious renewable hemicellulose [5]. Generally dietary NSPs remain indigestible and cannot be used as an energy source. Presence of β -glycosidic linkages makes cellulose indigestible for monogastric animals due to lack of cellulase in their gastrointestinal (GI) tract. Likewise, the other enzymes for NSP degradation (e.g., β -glucanases, β -xylanases) are also either sparse or absent in monogastric animals including fish [6]. Being incompletely soluble in water, NSPs enhance the viscosity of the digesta that affects physiology and ecosystem of the GI tract exercising anti-nutritive effects [4]. Therefore, removal of ANFs through implementation of processing techniques has been suggested to improve nutritive value of the crude oil-cakes and thereby incorporation of the processed oil-cakes at higher levels in the animal feeds satisfying to meet increasing demand to replace conventional

protein sources [7]. Studies advocated that processing of oil seed cakes through physical (e.g., water soaking and heat treatment) or biological (seed germination) [5] methods were not effectual in reducing the contents of ANFs. In contrast, solid state fermentation (SSF) has been evidenced with reduction in cellulose [8] and other ANFs [9] in the plant ingredients. Bio-processing of crop residues through microbial detoxification of agro-industrial wastes and enzyme production are the most feasible applications of the SSF [10]. Following oil extraction, recovery and reuse of nutrients in the oil-cakes by bioconversion through SSF may sustain economic viability of the agro-based oil producing industry. Correspondingly, effective use of the fermented oil-cakes for partial substitution of conventional animal protein sources (e.g., fishmeal) has been recommended in preceding studies [9,11].

Apart from de-activation of the ANFs, increase in the nutrient level through microbial synthesis of essential bio-molecules might be expected during the SSF [7]. In contrast, possible inclusion of harmful metabolites in the fermentation-product cannot be excluded during the SSF process. Therefore, it seemed reasonable to utilize microbial symbionts from fish gut in SSF-processing of the plant feedstuffs considering prospective application of the bio-processed substrate as fish feed ingredient [12,13]. Unlike the ruminants, fermentative nutrition is less emphasized in aquatic animals [14]. Although, diverse extracellular enzymes-producing gut microorganisms have been described in fish and it has been believed that enzymes produced by the gut associated symbiotic bacteria could assist in degradation and assimilation of complex plant feedstuffs within the GI tract of fish [15]. Cellulose and xyans being the major NSPs in plant feedstuffs, the

present study considered utilization of cellulase and xylanase-producing fish gut bacteria for bio-processing of oil-cakes through SSF. Several studies have reported occurrence of cellulase-producing microorganisms within the GI tracts of diverse fish species [for review see 15]. Production of cellulase in course of SSF has also been recorded by the bacterial strains (e.g., *B. subtilis* CY5, *B. circulans* TP3) isolated from fish gut [8,16]. In contrast, reports on xylanase production by fish gut microorganisms are scarce [17,18]. Though, production of xylanase by *Bacillus licheniformis* [19], *Bacillus sp.* AR-009 [20] and *B. pumilus* [21] isolated from non-fish sources have been documented previously under SSF.

In this perspective, the major objective of the presently reported study was to recycle the NSP-rich oil-cakes into value added foodstuffs. However, optimization of process parameters for scaling up cellulase and xylanase production by the microorganisms seemed to be imperative for efficient degradation of the NSPs under SSF. Therefore, the present study was undertaken (1) to explore the possibility of producing NSP-degrading enzymes (cellulase and xylanase) by the fish gut bacteria utilizing NSP-rich Linseed (*Linum usitatissimum*) oil-cake (LOC) as substrate, and finally (2) to appraise value addition of the substrate for its likely use as animal feed ingredient.

Materials and Methods

Microorganism and maintenance of culture

The bacteria used in the present study, the *Bacillus pumilus* LRF1X (KF640221) and *Bacillus tequilensis* HMF6X (KF640219) isolated from the proximal intestines of rohu, *Labeo rohita* and silver carp, *Hypophthalmichthys molitrix*, respectively, were described as efficient NSP-degrading strains in a previous report [18]. Following isolation of microbial strains from the gut of 6 freshwater carp species, potent NSP-degrading strains were determined through cellulase and xylanase producing abilities and identified by 16S rRNA partial gene sequence analyses [18]. The culture was grown and maintained on slants containing sterilized Nutrient Agar (NA) media. Inoculums of both the strains were prepared from a freshly raised 5-d-old slant culture in Nutrient Broth (37°C, 48 h) and inoculums thus prepared (6.5×10^7 cells mL⁻¹) were used for inoculation of the solid substrate medium.

Substrate selection

Dried and de-oiled groundnut oil-cake (GOC), mustard oil-cake (MOC), sunflower oil-cake (SOC), sesame oil-cake (SeOC) and linseed oil-cake (LOC) were collected from local market and used as solid substrates. Each of the oil-cakes was oven dried (80°C, 48 h), finely grounded in a laboratory mixer grinder and passed through a fine mesh (400 µm in diameter) sieve to obtain uniform particle size. The powdered samples were stored in a refrigerator (4°C) for use as substrates in solid state fermentation (SSF). Prior to analysis of cellulose and hemicellulose (xylan) contents, each solid substrate was made moisture free by drying at $100 \pm 5^\circ\text{C}$ initially for 30 min and further at 60°C until constant weight was obtained. Cellulose content was estimated with anthrone reagent following the method described by Updegraff [22]. Hemicellulose (xylan) was estimated after Goering and Van Soest [23]. LOC was selected as the solid-substrate due to the highest cellulose and xylan contents among the tested oil-cakes.

Optimization of SSF parameters for cellulase and xylanase production

Ten grams of dried and finely grounded substrate (LOC) was taken into each of the two sets of cotton plugged 250 mL Erlenmeyer flasks to evaluate cellulase and xylanase production by *B. pumilus* LRF1X (Set-I) and *B. tequilensis* HMF6X (Set-II). The moistening medium containing (g l^{-1}) K₂HPO₄ (0.15); MgSO₄ · 7H₂O (0.3) and (NH₄)₂SO₄ (2.0) was added as per required level to the substrate [24,25]. Flasks were autoclaved (121°C, 15 lbs, 20 min) and after proper cooling, sterilized substrates were inoculated (1 mL) separately with *B. pumilus* LRF1X and *B. tequilensis* HMF6X under aseptic conditions. Flasks were incubated (at 37°C for 72h, otherwise mentioned) in stationary condition with mechanical agitation by sterilized glass-rods at 12 h intervals. Each step of the assessment was carried out in triplicate. The protocol adopted for optimization of different process parameters was to assess the effect of an individual parameter and to incorporate it at the optimized level before optimizing the next parameter.

The physicochemical parameters studied were: initial moisture content (10%-90% moistening media, v/w), incubation temperature (25°C-50°C), initial pH of the moistening media (pH 5-9), inoculum size (1-10% v/w, at an interval of 1% with 48h culture containing 6.5×10^7 cells mL⁻¹), and NaCl (1%-5% w/w). Further, different surfactants (1% v/w), viz., Tween 20, Tween 40, Tween 80 and Dimethyl sulfoxide (DMSO), carbon sources (1% w/w; glucose, sucrose, lactose, maltose, fructose and starch) and inorganic or organic nitrogen sources (1% w/w; ammonium sulfate, ammonium nitrate, ammonium chloride, yeast extract, peptone and tyrosine) were also optimized. The selected carbon and nitrogen sources were varied within a narrow range (1-5%) to optimize collective production of both the enzymes. Following optimization of various process parameters, a time course study was carried out for 10 days (considering 24h as 1 day) with both SSF batches incorporating all parameters at the optimized level. Cellulase and xylanase production by the bacterial strains, and subsequent degradation of cellulose and xylan in the substrate were evaluated at an interval of 2 days [26,27].

Enzyme extraction

Extraction of the crude enzymes was carried out separately from the fermented substrate for evaluation of cellulase and xylanase production by the bacterial strains (*B. pumilus* LRF1X and *B. tequilensis* HMF6X) during SSF. For cellulase assay, crude enzyme was extracted following Pandit and Maheswari [26] with minor modifications. The fermented material (5 g) was mixed thoroughly with 50 mL of 1 M phosphate buffer (pH 6.5) (except in case of pH optimization, where distilled water was used) and left on a shaker incubator (Lab. companion, SL-300R) at 15°C for 1 h with continuous shaking at 150 rpm. The fermented samples were then filtered through muslin cloth as cellulase was strongly absorbed by the filter papers that contain cellulose [27]. The filtrates were centrifuged at 4°C for 20 min at 10,000 rpm in a refrigerated centrifuge and the cell free supernatant thus obtained was collected as crude enzyme for cellulase assay.

Crude enzyme for xylanase assay was extracted following Banu and Ingale [28] with minor modification. Sodium phosphate buffer (0.1 M, pH 7.0) was added with the fermented solid substrate (5g) and kept on a shaker incubator (15°C, 150 rpm, 1 h). The crude enzyme was then separated by filtration through filter paper (Whatman No. 1). The filtrate was centrifuged at 4°C for 20 min at 10,000 rpm in a

refrigerated centrifuge and the cell free supernatant was collected as crude enzyme for xylanase assay.

Cellulase and xylanase assay

Cellulase activity was measured according to the method of Denison and Koehn [29]. The production of reducing sugar (glucose) from the carboxymethylcellulose substrate was measured at 540 nm by the di-nitro-salicylic acid (DNSA) method using glucose as the standard. Unit activity (U) of cellulase was defined as the μg of glucose liberated mL^{-1} enzyme extract min^{-1} . Quantitative assay of xylanase activity was measured after Bailey et al. [30] through DNSA method using birch-wood xylan (1%) as the substrate. Unit activity (U) of xylanase was expressed as mg of D-xylose liberated mL^{-1} enzyme extract min^{-1} .

Analysis of proximate composition and antinutrients

Cellulose and hemicellulose (xylan) contents of raw and fermented LOC were analyzed following the methods of Updegraff [22] and Goering and Van Soest [23], respectively. Proximate composition of raw and fermented LOC were analyzed following the standard methods of AOAC [31]: crude protein ($\text{N}\% \times 6.25$) by micro Kjeldahl digestion and distillation, lipid was determined by extracting the residue with 50-60°C petroleum ether in a Soxhlet apparatus, crude fiber was determined as loss on ignition of dried lipid free residue after digestion with 1.25% H_2SO_4 and 1.25% NaOH. Ash content of the sample was determined by ignition of samples at 550°C in a muffle furnace to constant weight. Nitrogen-free extract (NFE) was computed by taking the sum of values for crude protein, crude lipid, ash, crude fibre and moisture and subtracting this from 100 [32]. Gross energy of the raw and fermented samples was measured with a bomb calorimeter (Lab-X, Kolkata, India). Total free amino acids and fatty acids were measured according to Moore and Stein [33] and Cox and Pearson [34], respectively. Among the antinutritional factors, tannin and phytic acid were determined by biochemical methods described by Schanderi [35] and Vaintraub and Lapteva [36], respectively. Trypsin inhibitor activity was determined according to Smith et al. [37].

Analysis of amino acid composition of raw and fermented linseed oil-cake

The amino acid profile of the raw and fermented substrates (Set-I) were determined by a high performance liquid chromatography (HPLC) system (Agilent Technologies-1260 Infinity) equipped with an ion-exchange/reversed-phase column (Zorbax Eclipse XDB-C18, Agilent Technologies). Samples were hydrolyzed with 6N HCl at 120°C for 24 h [38], filtered through 0.45 μm filtration assembly and analyzed after pre-column derivitization of amino acids with an autosampler. The primary amino acids were derivitized with O-phthalaldehyde (OPA) and the secondary amino acids were derivitized with fluorenylmethyl chloroformate (FMOC) before injection [39]. Each sample was run in triplicate. Chromatograms of the samples obtained were matched for their retention time with those of the standard amino acid mixture and quantification was achieved mathematically.

For tryptophan content, samples were hydrolyzed by heating at about 110°C in 6 N NaOH (20 h) and determined spectrophotometrically following the method described by Sastry and Tammuru [40].

Statistical analysis

All experiments were performed in triplicate and the mean values were reported along with standard error (mean \pm SE, $n=3$). All the statistical analyses [One-way ANOVA] of the data were performed according to Zar [41] using SPSS Ver10 [42] software.

Results and Discussion

An attempt has been made in the present study for bio-processing of nutrient rich oil-cakes through degradation of NSPs. SSF was carried out in view of amelioration of nutritive value in LOC, as well as cellulase and xylanase production utilizing LOC as the solid substrate. SSF has been described as the most preferred way for microbial cellulase production due to its lower capital investment and lower operating cost [43]. Xylanase, another important industrial enzyme may also be successfully produced though SSF [44], although very few reports are available on the production of xylanases by bacterial systems under SSF [19,20]. Abundance of cellulase-producing bacteria has been documented in the GI tracts of grass carp, *C. idella* [16,45], common carp, *C. carpio* and silver carp, *H. molitrix* [16], rohu, *L. rohita* [46,47], catla, *C. catla* and mrigal, *C. mrigala* [47], and bata, *L. bata* [48,49]. In contrast, reports on xylanase-producing fish gut microorganisms are scanty [17,18]. In the present study, optimization of the important physical, chemical and nutritional parameters were performed that influenced cellulase and xylanase production as well as bio-processing of the LOC by *B. pumilus* LRF1X and production *B. tequilensis* HMF6X, which were autochthonous to fish. In vitro processing by autochthonous microbiota has been suggested as an effective strategy as the organism itself, and their metabolites would not harm the fish providing the basis for mutual relationship [12].

Selection of substrate

Five oil-cakes were primarily evaluated for cellulose and hemicellulose (xylan) contents. The results are presented in table 1. It was evident that cellulose content was the highest in LOC (17.51%, dry weight), which was followed by SOC (16.98%). Xylan is the most common hemicellulose as well as the major non-cellulosic cell wall polysaccharide of angiosperms, grasses, cereals and seeds, where they exist in diverse compositions and structures [50]. In comparison to the oil-cakes tested, hemicellulose (xylan) content was also found to be the maximum (13.02%) in LOC. Consequently, LOC was used as solid substrate in subsequent studies.

Oil-cakes*	Cellulose	Hemicellulose (Xylan)
GOC	15.13 \pm 0.71 ^b	12.98 \pm 0.58 ^b
MOC	11.32 \pm 0.48 ^a	10.11 \pm 0.44 ^a
SOC	16.98 \pm 0.73 ^c	12.49 \pm 0.55 ^b
LOC	17.51 \pm 0.87 ^c	13.02 \pm 0.66 ^b
SeOC	15.55 \pm 0.66 ^b	11.12 \pm 0.49 ^a

Table 1: Contents of cellulose and hemicellulose (xylan) in different oil-cakes (on % dry matter basis). Values are means \pm SE of three determinations. Values with the same superscript in the same row are not significantly different ($P<0.05$) from each other.*GOC, groundnut oil-cake; MOC, mustard oil-cake; SOC, sunflower oil-cake; LOC, linseed oil-cake; SeOC, sesame oil-cake

Effect of initial moisture content

Moisture content of the substrate is one of the critical factors influencing the outcome of SSF as the microorganisms grow near the surface of the solid substrate particles associated with low moisture content [51]. There was gradual increase in production of both the

enzymes by both the bacteria with increase in the moisture content up to certain level when incubated at 37°C for 72 h. The maximum cellulase (65.27 ± 1.06 U g⁻¹) and xylanase (25.93 ± 0.87 U g⁻¹) production by *B. pumilus* LRF1X was obtained at 60% moisture level (Figure 1 A).

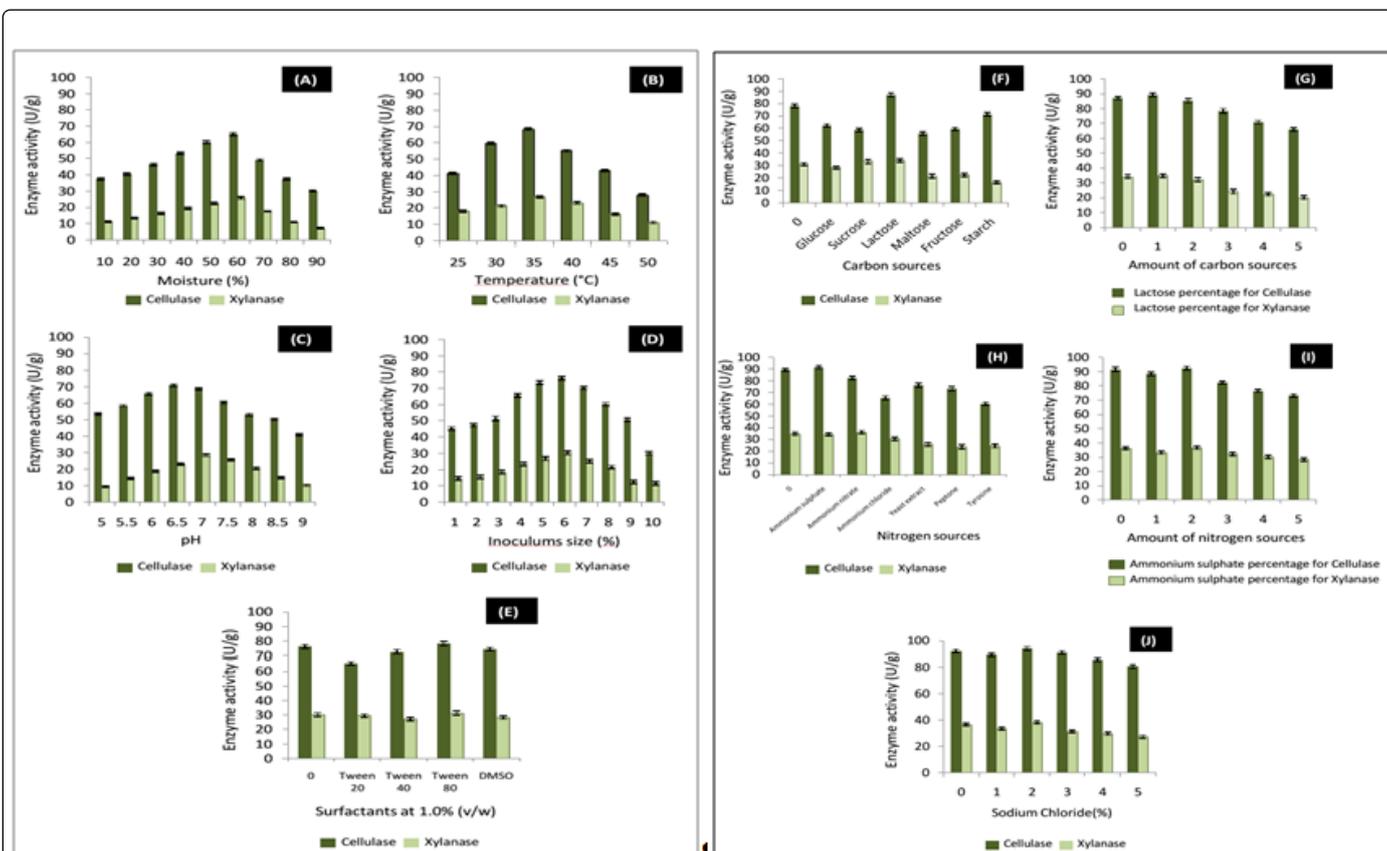


Figure 1: Effect of (A) moisture (%), (B) temperature (°C), (C) pH, (D) inoculum size (%) and (E) surfactants (1.0% v/w) on cellulase and xylanase production by *Bacillus pumilus* LRF1X (KF640221). Effect of (F) carbon sources, (G) varying levels of the selected carbon source, (H) nitrogen sources, (I) varying levels of the selected nitrogen sources and (J) varying levels of the sodium chloride on cellulase and xylanase production by *Bacillus pumilus* LRF1X (KF640221).

The strain *B. tequilensis* HMF6X also produced the highest cellulase (51.36 ± 0.97 U g⁻¹) and xylanase (24.37 ± 0.85 U g⁻¹) with 60% moisture in the SSF media (Figure 2A). Above this, production of both the enzymes was found to decrease. This could be due to reduced porosity and oxygen deprivation in the substrate with increased moisture content leading to lesser biomass and enzyme production [52]. One-way ANOVA revealed that variation in the production of both the enzymes at different initial moisture levels were statistically significant (P < 0.05).

Effect of Different Temperature

The incubation temperature is another vital factor regulating the enzyme synthesis [53]. Incubation temperature also influences the transport of enzymes across the membrane, thereby affecting the enzyme yield [21,54]. The optimum temperature for production of cellulase and xylanase by both, *B. pumilus* LRF1X (Figure 1B) and *B. tequilensis* HMF6X (Figure 2B) were observed to be 35°C. The maximum cellulase and xylanase yield at optimum temperature during

SSF of LOC were 68.39 ± 1.12 U g⁻¹ and 26.74 ± 0.91 U g⁻¹, respectively, by *B. pumilus* LRF1X. Whereas, in case of *B. tequilensis* HMF6X the highest cellulase and xylanase produced at optimum temperature were 53.67 ± 0.95 U g⁻¹ and 26.42 ± 0.88 U g, respectively. The result indicated that cellulase was highly sensitive towards temperature as cellulase yield by *B. pumilus* LRF1X was reduced to 28.18 ± 0.89 U g⁻¹ at 50°C. Most of the biological processes are operated in relatively narrow range of temperature [55]. Temperature plays important role in obtaining a good cellular viability, best enzymatic production and extracellular protein synthesis [56]. A fungal strain, *Alternaria alternata* was accounted for maximum cellulase production at 35°C under SSF [57], which was consistent with our observation. Although, Maurya et al. [58] and Bhaumik et al. [59] reported 30°C as optimum for maximum cellulase production under SSF by *Trichoderma reesei* and *Trametes hirsute*, respectively. Whereas, thermophilic *B. licheniformes* gave the best result for cellulase production at 50°C under submerged fermentation conditions.

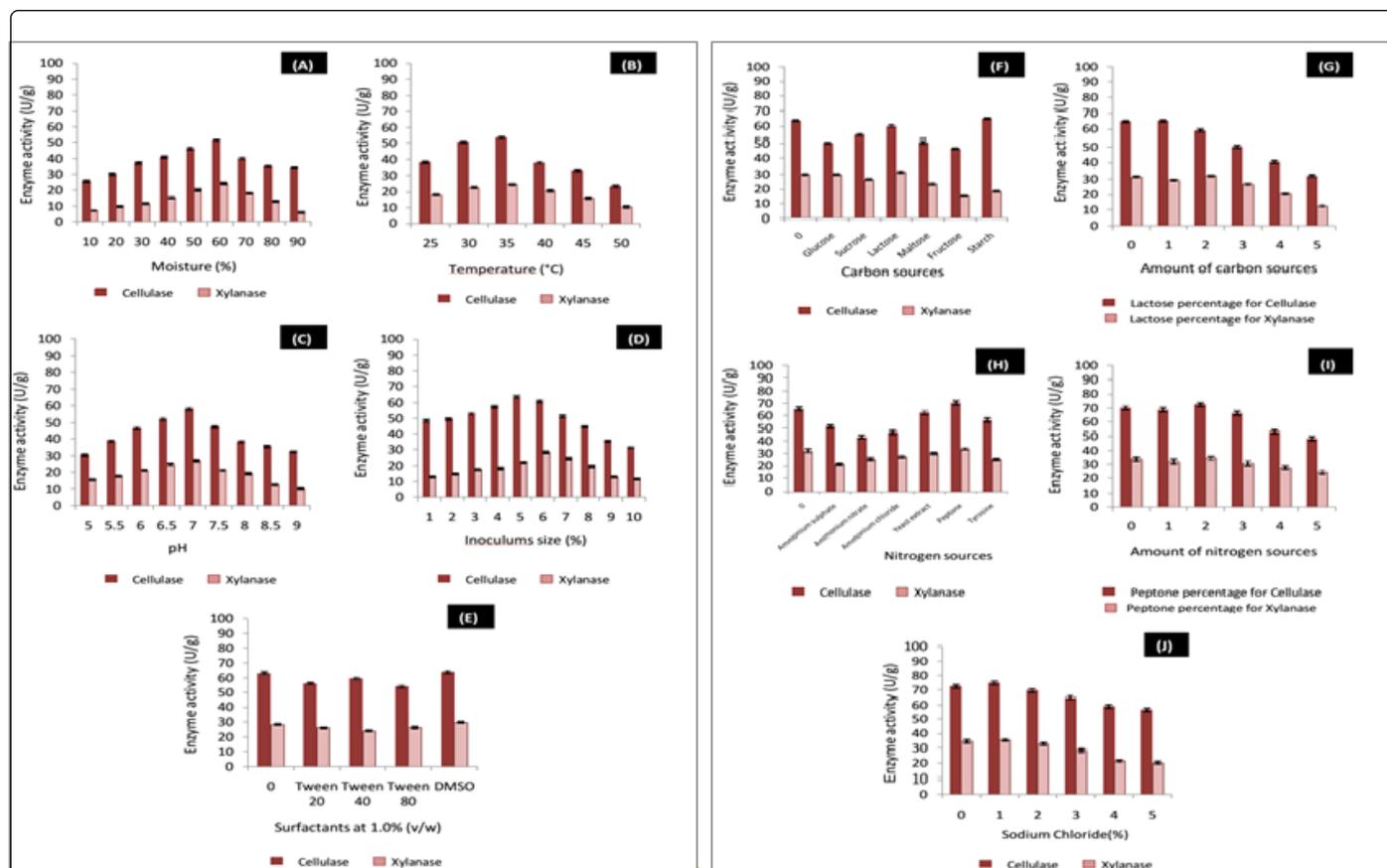


Figure 2: Effect of (A) moisture (%), (B) temperature (°C), (C) pH, (D) inoculum size (%) and (E) surfactants (1.0% v/w) on cellulase and xylanase production *Bacillus tequilensis* HMF6X (KF640219). Effect of (F) carbon sources, (G) varying levels of the selected carbon source, (H) nitrogen sources, (I) varying levels of the selected nitrogen sources and (J) varying levels of the sodium chloride on cellulase and xylanase production by *Bacillus tequilensis* HMF6X (KF640219).

Previous study with *B. subtilis* BS04 resulted in the highest xylanase production at 35°C under submerged fermentation. The optimum temperature for maximum xylanase production by *B. subtilis* ASH in SSF was found to be 37°C [60], which was in close agreement with our observation. The similar optimum temperature ranges for xylanase production in SSF were reported by Beg et al. [61] and Battan et al. [5]. In contrast, the highest xylanase production by thermophilic *B. licheniformis* A99 was observed at 50°C [19].

Effect of Different pH

Optimization of the initial pH of the medium seemed to be essential in SSF as extracellular enzymes are stable only at a particular pH and there may be quick denaturation at lower or higher values [58,60]. The effect of pH of the moistening media on production of both the enzymes by *B. pumilus* LRF1X and *B. tequilensis* HMF6X have been depicted in figure 1C and figure 2C, respectively. It was revealed that pH 6.5 was optimum for cellulase production ($70.95 \pm 1.31 \text{ U g}^{-1}$) by *B. pumilus* LRF1X, which was followed by pH 7.0 ($68.58 \pm 1.28 \text{ U g}^{-1}$). While, pH 7.0 was noticed as optimum for xylanase production ($28.53 \pm 0.84 \text{ U g}^{-1}$) by the strain *B. pumilus* LRF1X. Considering this, the moistening agent with pH 7.0 was used for SSF of the LOC. Within the tested pH range, pH 7.0 was optimum for both cellulase ($58.14 \pm 0.88 \text{ U g}^{-1}$) and xylanase ($26.95 \pm 0.71 \text{ U g}^{-1}$) production by *B. tequilensis*

HMF6X. In addition, both the bacterial strains could produce considerable enzymes up to pH 7.5.

Production of cellulases by diverse bacterial species was reported in the pH range of 4.0 to 9.0, with maximum activity around pH 7.0 [62]. Maximum xylanase production by *B. pumilus* AB-1 under SSF was observed at pH 7.0, which was in accordance with our finding [28]. In contrary, optimum production of cellulase and xylanase in acidic pH (3.7-5) has been recorded by diverse Bacilli under SSF [63]. Furthermore, optimum cellulase and xylanase production accomplished in the present study around the initial pH 7 might be due to the fact that the bacterial symbionts used in the SSF were isolated from the gut of agastric carps (rohu, *Labeo rohita* and silver carp, *Hypophthalmichthys molitrix*), and the bacteria were adapted to the neutral or slightly alkaline pH therein [18].

Effect of inoculum size

The initial inoculum level in the media is a vital factor in fermentation process [64] Both the enzyme activities gradually increased with increase in inoculum size for *B. pumilus* LRF1X up to 6.0% (v/w), and thereafter declined. Cellulase ($76.17 \pm 1.08 \text{ U g}^{-1}$) and xylanase ($30.27 \pm 0.95 \text{ U g}^{-1}$) production was highest at inoculum size 6.0% (v/w) (Figure 1 D). For *B. tequilensis* HMF6X, the maximum

yield of cellulase ($63.25 \pm 0.76 \text{ U g}^{-1}$) was achieved with 5.0% (v/w) inoculums, whereas 6.0% inoculums size (v/w) also resulted in considerable cellulase ($60.89 \pm 0.68 \text{ U g}^{-1}$) and maximum xylanase ($28.34 \pm 0.65 \text{ U g}^{-1}$) production (Figure 2 D). Considering these facts, 6.0% (v/w) inoculum size of *B. tequilensis* HMF6X was used during SSF-processing of LOC. An inoculum size varying between 4-6% has been described as optimum for cellulase production by diverse microorganisms [65], which was close to our observation. *Aspergillus flavus* AT-2 and *Aspergillus niger* AT-3 revealed maximum cellulase production under SSF with an inoculum size of 5% [66]. In contrast, *B. licheniformis* A99, *B. pumilus* ASH 7411 and *B. subtilis* ASH were reported to produce highest xylanase when as high as 15% inoculum level was used [5,19,60]. Likewise, a 10% inoculum was also documented as optimum for production of xylanase by *B. megaterium* under SSF [67].

Optimum inoculum size may vary with organisms and the substrates used in SSF. At certain level, enzyme production could decrease because of depletion of nutrients and greater competition for nutrient uptake due to the increased biomass, which would diminish the metabolic activity [68]. Therefore, equilibrium between the proliferating microbial biomass and existing substrate material should be sustained to yield maximum enzyme [69].

Effect of different surfactants

Surfactants have been reported to influence the growth and extracellular enzyme production of the microorganisms and their effects might vary from enzyme to enzyme, even from organism to organism [13]. In the present study, highest cellulase ($78.15 \pm 1.18 \text{ U g}^{-1}$) and xylanase ($31.17 \pm 0.66 \text{ U g}^{-1}$) activities by *B. pumilus* LRF1X was revealed with the supplementation of Tween 80 into the SSF medium (Figure 1E). Whereas, DMSO gave maximum cellulase ($63.84 \pm 1.04 \text{ U g}^{-1}$) and xylanase ($29.77 \pm 0.58 \text{ U g}^{-1}$) yields by *B. tequilensis* HMF6X, which are depicted in Figure 2E. Stimulatory effect of Tween 80 on cellulase production by *Penicillium sp.* has been indicated [70]. Tween 80 was also reported to give positive effects on the production of xylanase by *B. subtilis* and *Aspergillus awamori* [71]. However, modulation of microbial xylanase activity by DMSO has not been authentically reported previously. Most possibly the surfactants increase the cell membrane permeability that in turn lead to simultaneous increase in the secretion and extraction of enzymes from SSF [72].

Effect of various carbon sources and their amount

Optimization of various supplemented carbon sources (1%) revealed that lactose was the most effective carbon source for both cellulase ($87.14 \pm 1.21 \text{ U g}^{-1}$) and xylanase ($34.16 \pm 0.62 \text{ U g}^{-1}$) production by *B. pumilus* LRF1X (figure 1F). While, starch ($65.28 \pm 1.07 \text{ U g}^{-1}$) induced the highest cellulase yield by *B. tequilensis* HMF6X, which was followed by lactose ($60.12 \pm 0.95 \text{ U g}^{-1}$) (figure 2F). Lactose supplementation also resulted in maximum xylanase production ($31.48 \pm 0.61 \text{ U g}^{-1}$) by *B. tequilensis* HMF6X (Figure 2F). Further analyses with varying lactose levels revealed that 1% (w/v) lactose was optimum for cellulase production by both the strains ($89.23 \pm 1.18 \text{ U g}^{-1}$ and $65.36 \pm 0.91 \text{ U g}^{-1}$, respectively), and xylanase production ($34.87 \pm 0.64 \text{ U g}^{-1}$) by *B. pumilus* LRF1X (Figures 1G and 2G). However, 2% lactose supplementation brought about maximum xylanase yield ($32.11 \pm 0.61 \text{ U g}^{-1}$) by *Bacillus tequilensis* HMF6X (Figure 2 G). Further increase in the carbon level in both cases diminished enzyme production.

In general, cellulases were described as inducible enzymes and were shown to be induced by the presence of soluble saccharides [70]. In accordance to our results, improved cellulase production through lactose supplementation was reported by *B. subtilis* [73]. The mechanism of lactose induced increase in cellulase yield was thought to be on account of intracellular galactose-1-phosphate levels [74]. However, reports on the effect of different carbon sources in the production of microbial xylanases were conflicting with contradictory results. Supplementary carbon sources, such as starch, sucrose, maltose, lactose or glucose at 1% were reported to improve the production of xylanase by *B. megatherium* [75]. Our study revealed only marginal increase in xylanase production through inclusion of lactose and sucrose as additional carbon sources. In contrast, xylanase production by *Bacillus sp.* AR-009 grown on wheat bran was repressed upon addition of lactose, glucose and sucrose [20]. Catabolite repression by glucose and/or xylose was also observed in many other *Bacillus spp.* [5,60]. In the present study, repressed xylanase production was noticed through inclusion of glucose, maltose, starch and fructose in the SSF media.

Effect of various nitrogen sources and their amount

Nitrogen sources are important nutrients for enzyme production. Among all the organic and inorganic nitrogen sources tested, the highest cellulase ($91.53 \pm 1.33 \text{ U g}^{-1}$) and xylanase ($36.05 \pm 0.62 \text{ U g}^{-1}$) production by *B. pumilus* LRF1X was achieved with supplementation of ammonium sulphate and ammonium nitrate, respectively (Figure 1 H). Inclusion of ammonium sulphate evidenced the second best xylanase production ($34.11 \pm 0.55 \text{ U g}^{-1}$) by *B. pumilus* LRF1X. Further study revealed that 2.0% (w/v) supplementation of ammonium sulphate was optimum for production of both cellulase ($92.36 \pm 1.28 \text{ U g}^{-1}$) and xylanase ($36.47 \pm 0.56 \text{ U g}^{-1}$) by *B. pumilus* LRF1X. (Figure 1 I). Another strain, *B. tequilensis* HMF6X resulted in the highest cellulase ($70.16 \pm 0.95 \text{ U g}^{-1}$) and xylanase ($33.75 \pm 0.51 \text{ U g}^{-1}$) production when supplemented with peptone as an additional nitrogen source (figure 2H). Further study revealed 2% (w/w) peptone as optimum concentration for cellulase ($72.33 \pm 1.05 \text{ U g}^{-1}$) and xylanase ($34.33 \pm 0.54 \text{ U g}^{-1}$) production by *B. tequilensis* HMF6X (Figure 2 I). Supplementation of additional nitrogen sources provided enhancement of cellulase and xylanase production in several previous studies. In conformity with our observation, the maximum cellulase production by *B. subtilis* [73] and *Aspergillus spp.* [66] were observed with ammonium sulphate supplementation, which support our observation. Although, improved cellulase production by *Penicillium sp.* with inclusion of ammonium nitrate was also reported [70]. Similar with our results obtained in *B. tequilensis* HMF6X, peptone caused was described to cause marginal increase in xylanase activity by *B. subtilis* ASH [60].

Effect of NaCl

In the present study, 2% NaCl (w/w) supplementation showed highest cellulase ($94.15 \pm 1.59 \text{ U g}^{-1}$) and xylanase ($37.96 \pm 0.68 \text{ U g}^{-1}$) production by *Bacillus pumilus* LRF1X (Figure 1J). However, *B. tequilensis* HMF6X produced the maximum cellulase ($74.78 \pm 1.08 \text{ U g}^{-1}$) and xylanase ($35.49 \pm 0.56 \text{ U g}^{-1}$) with 1.0%, NaCl (w/w) supplementation (figure 2J). Previously, enhanced cellulase production by *B. pumilus* was observed with 2.5% NaCl supplementation [64].

Effect of incubation period on cellulase and xylanase production and on cellulose and xylan degradation

Following optimization of the various process parameters, a time course study was conducted with LOC as substrate to notice the cumulative effect of various parameters. Enzyme production increased gradually with incubation time and maximum cellulase ($95.25 \pm 1.14 \text{ U g}^{-1}$) and xylanase ($38.02 \pm 0.53 \text{ U g}^{-1}$) production by *B. pumilus* LRF1X was obtained after 8 days (Figure 3).

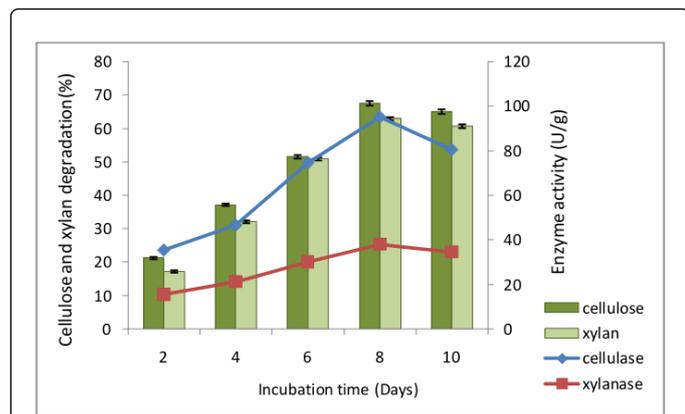


Figure 3: Effect of incubation time on cellulase and xylanase production along with cellulose and xylan degradation by *Bacillus pumilus* LRF1X (KF640221).

Concentration of NSPs in the substrate reduced gradually as fermentation progressed. Degradation of cellulose (67.61%) and xylan (62.98%) in the LOC was observed through SSF by *B. pumilus* LRF1X after 8 days. Degradation of the NSPs did not increase further during the last two days of incubation. Similarly, the highest yields of cellulase ($75.02 \pm 0.84 \text{ U g}^{-1}$) and xylanase ($36.15 \pm 0.59 \text{ U g}^{-1}$) by *B. tequilensis* HMF6X were achieved after 8 days under optimized conditions (Figure 4), that lead to degradation of cellulose (60.37%) and xylan (54.92%) in the solid substrate (LOC). Enzyme yield declined during

further incubation probably due to reduced nutrient level in the medium. Otherwise, it could also be the result of poisoning and denaturation of the enzyme by interaction with other components in the medium [76]. Degradation of cellulose and xylan mostly ceased after day 8, which might be in consequence of decreased enzyme concentration.

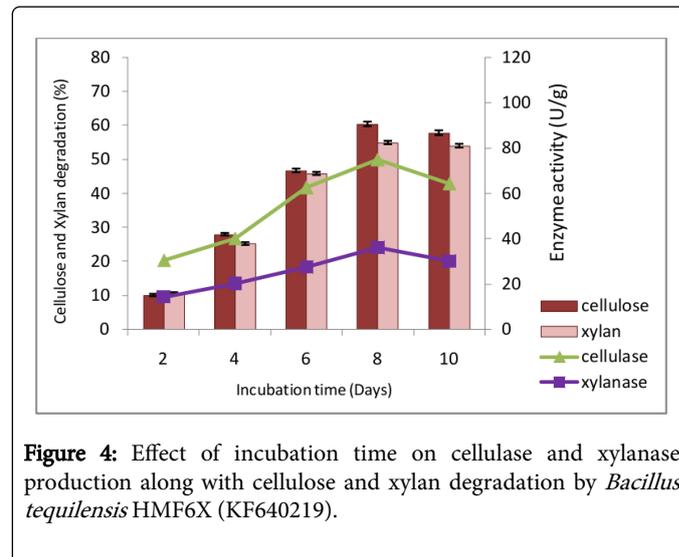


Figure 4: Effect of incubation time on cellulase and xylanase production along with cellulose and xylan degradation by *Bacillus tequilensis* HMF6X (KF640219).

Effect of fermentation on proximate composition of LOC

Waste utilization through the generation of value added by-products is the major benefit for using agro-industrial residues in SSF. Analyses of proximate composition in the LOC following SSF at optimal conditions by the fish gut isolates *B. pumilus* LRF1X (Set-I) and *B. tequilensis* HMF6X (Set-II) revealed that there were minor improvement in the contents of crude protein, lipid, ash, total free amino acids and fatty acids as compared to the raw LOC (Table 2). SSF of LOC was effective in significantly

Parameters	Raw LOC	Fermented LOC			
		Set-I		Set-II	
		SSF with LRF1X	%Increase (↑)/Reduction (↓)	SSF with HMF6X	%Increase (↑)/Reduction (↓)
Dry matter*	92.26 ± 0.53 ^a	96.61 ± 0.48 ^b	4.71↑	94.85 ± 0.55 ^b	2.81↑
Moisture	7.74 ± 0.31 ^c	3.39 ± 0.26 ^a	56.2↓	5.15 ± 0.29 ^b	33.46↓
Crude protein	34.09 ± 0.81 ^a	36.86 ± 0.88 ^b	8.13↑	36.18 ± 0.78 ^b	6.13↑
Crude lipid	13.84 ± 0.53 ^a	15.82 ± 0.24 ^b	14.3↑	15.94 ± 0.25 ^b	15.17↑
Ash	6.18 ± 0.23 ^a	6.89 ± 0.19 ^b	11.49↑	6.77 ± 0.21 ^b	9.54↑
Crude fibre	7.61 ± 0.35 ^c	4.17 ± 0.22 ^a	45.2↓	4.94 ± 0.26 ^b	35.08↓
NFE	30.54 ± 0.76 ^a	32.87 ± 0.78 ^a	7.63↑	31.02 ± 0.73 ^a	1.89↑
Total carbohydrate#	38.15 ± 0.45 ^b	37.04 ± 0.41 ^a	2.91↓	35.96 ± 0.49 ^a	5.74↓
Cellulose	17.51 ± 0.87 ^c	5.67 ± 0.21 ^a	67.61↓	6.94 ± 0.26 ^b	60.37↓

Hemicellulose (Xylan)	13.02 ± 0.66 ^c	4.82 ± 0.24 ^a	62.98↓	5.86 ± 0.23 ^b	54.97↓
Gross energy (kcal/g)\$	4.81 ± 0.05 ^a	4.89 ± 0.04 ^a	1.65↑	4.86 ± 0.05 ^a	1.03↑
Total free amino acids	0.23 ± 0.04 ^a	0.31 ± 0.02 ^b	34.78↑	0.29 ± 0.04 ^a	26.08↑
Total free fatty acids	1.46 ± 0.07 ^a	1.61 ± 0.03 ^b	10.27↑	1.58 ± 0.05 ^a	8.21↑
Tannin	2.31 ± 0.06 ^b	1.47 ± 0.06 ^a	36.37↓	1.53 ± 0.08 ^a	33.76↓
Phytate	1.14 ± 0.03 ^c	0.73 ± 0.02 ^a	35.96↓	0.89 ± 0.04 ^b	21.92↓
Trypsin inhibitor	1.73 ± 0.07 ^b	0.63 ± 0.05 ^a	63.58↓	0.61 ± 0.04 ^a	64.74↓

Table 2: Proximate composition of raw and fermented linseed oil-cake (on % dry matter basis). Values are means ± SE of three determinations. Values with the same superscript in the same row are not significantly different (P<0.05) from each other. *Includes organic matter and ash. Dried to constant weight at 100°C. #Total carbohydrate=NFE+Crude fibre. \$kcal=Kilocalorie (Unit of energy).

(P<0.05) reducing the contents of NSPs (both, cellulose and hemicellulose), crude fibre and other ANFs, e.g., tannins, phytic acid and trypsin inhibitor. Apparently, it was revealed that SSF by *B. pumilus* LRF1X (Set-I) was more effective than *B. tequilensis* HMF6X (Set-II) in improving the nutrient levels and reducing the ANFs including cellulose and hemicellulose (xylan) contents of LOC, except for lipid. SSF, as a consequence, presumably assisted in processing of LOC that in turn led to improvement of its nutritive value. During fermentation, ameliorating the nutrient level through microbial synthesis might be expected [7,9], which were in agreement with the present report. Increased level of crude protein, lipid, free amino acids, and free fatty acids in fermented oil-cakes in comparison to the raw substrate was consistent to the findings of Ghosh and Mandal [9], Ramachandran et al., [11], Bairagi et al. [16] and Khan and Ghosh.

Amino acids	Raw LOC		Fermented LOC	
	% dry matter	% crude protein	% dry matter	% crude protein
Arginine	3.31	9.73	4.01	10.88 (↑)
Histidine	1.16	3.41	1.67	4.53 (↑)
Isoleucine	1.54	4.53	1.79	4.87 (↑)
Leucine	1.82	5.33	1.8	4.89 (↓)
Lysine	1.31	3.85	1.16	3.16 (↓)
Methionine	0.62	1.82	0.9	2.45 (↑)
Phenylalanine	1.93	5.68	2.55	6.92 (↑)
Threonine	1.86	5.47	2.47	6.71 (↑)
Tryptophan	0.77	2.25	1.09	2.96 (↑)
Valine	1.57	4.62	1.9	5.17 (↑)
Cystine	0.76	2.23	0.89	2.41 (↑)

Table 3: Amino acid composition of raw and fermented linseed oil-cake in Set-I (fermented with *B. pumilus* LRF1X). Each value represents mean of three determinations. Amino acid contents are expressed as both, % dry matter and % crude protein. Increase (↑) or decrease (↓) in the amino acid contents of fermented LOC is indicated in the parenthesis.

Effect of fermentation by *B. pumilus* LRF1X on amino acid composition

Our result is consistent with the report of Ghosh and Mandal [9], where improvement of amino acid balance in the groundnut oil-cake has been reported through SSF. Plant feedstuffs are usually deficient in the sulphur containing amino acids, viz., lysine, cystine and methionine. Therefore, increase in the contents of methionine and cystine in consequence of SSF could be worth to mention.

Conclusions

The results of the present study suggest that adoption of SSF as a bio-processing strategy might contribute to enhance the nutritive value of the oil-cakes. Value addition in the bio-processed LOC became apparent from increase in the level of nutrients (crude protein, lipid etc.) and decrease in the contents of ANFs like NSPs, tannin, phytic acid and trypsin inhibitor. Moreover, SSF by *B. pumilus* LRF1X resulted in improved amino acid profile in the SSF-processed LOC. The bio-processed LOC produced in the present study might be included in the diets for carps or other monogastric animals at higher ratios replacing the conventional protein sources, that would successively reduce the feed cost and feed-related waste outputs. Thus, adoption of SSF as a bio-processing technique might contribute to enhance the nutritive value of oil-cakes or other agro-industrial wastes. Present study also reported cellulase and xylanase producing ability of the two autochthonous fish gut bacteria, *B. pumilus* LRF1X and *B. tequilensis* HMF6X under SSF using LOC as the substrate. Therefore, concurrent production of NSP-degrading enzymes together with generation of bio-processed solid substrate might be of great economic viability for application of this bio-processing strategy at an industrial scale. Furthermore, possibilities for their direct inclusion of the NSP-degrading fish gut bacteria as probiotics could be emphasized in forthcoming studies to improve nutrient utilization in monogastric animals fed plant feedstuffs incorporated diets.

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