Nutritional Fortification of Sunflower Meal by *Bacillus Subtilis ATCC PTA-6737* Fermentation

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Abstract

Sunflower meal with 3 mm particle size (SFM) with 66 or 80% moisture content was inoculated with $1x10^7$ cfu (colony forming unit/g) of *Bacillus subtilis ATCC PTA-6737* and fermented for 0, 24, 48 and 72 h. The pH was increased from 5.75 to 9.1 of SFM fermented at both moisture levels with no significant changes in organic acid production. The bacterial growth was peaked at 24 h. Dry matter and crude fibre contents of SFM decreased by 10-13 and 18%, respectively. In contrary, the amount of crude protein, crude ash and soluble amino acid nitrogen increased by 36, 61% and 145%, respectively, with no regard to the effect of moisture content. Phytic acid was degraded up to 42% at both level of moisture content. In conclusion, SFM was enriched with ash and protein and lowered in fibre and phytic acid contents, and can be used as alternative feed material in animal nutrition.

Keywords: *Bacillus subtilis*, sunflower meal, fermentation, nutrient fortification

INTRODUCTION

Sunflower meal (SFM) has been used in animal nutrition as a second plant protein source after soybean meal (SBM), but its use is limited due to its high fibre content and some antinutrional factors (ANFs), especially for feeding some poultry species (Sangsoponjit *et al.,* 2017).Solid state fermentation (SSF) using safe microorganisms is a process of fermentation where solid materials are immersed in water, and the production yield of several products such as enzymes, organic acids, aromatic and antimicrobial agents is greater than liquid state fermentation process (Raimbault, 1998; Singhaniaa *et al.,* 2009; Afşin, 2010; Özşölen, 2010; Ravichandran and Vimala, 2012 and Mukherjee *et al.,* 2016). For instance, SFM can be used as fermenting substrate in microbial fermentation process to produce protease (Haq and Mukhtar, 2004) and lipase enzymes (Karakoç, 2006). *Bacillus subtilis* is a gram-positive bacteria, found in soil and gastrointestinal tract (Yonsel, 2010) and widely used in many industrial applications (Yonsel, 2010; Constantinscu and Petruta, 2015). Moreover, several species of *Bacillus* have been used to produce amylase (Baysal *et al.*, 2002; Choubane *et al.,* 2015) and alkaline protease enzyme (Uyar and Baysal, 2004; Patel *et al.,* 2005; Prakasham *et al.,* 2007; Mukherjee *et al.,* 2008).

Enzymatic hydrolysis or defatting process of SFM was earlier shown to increase the crude protein levels (Cai *et al.,* 1996). Recently, microbial fermentation of feed materials including SFM with *Saccharomyces cerevisiae* or *Bacillus subtilis* has been shown to increase the contents of crude protein, lipid and some essential amino acid; all these were associated with reduced crude fibre content and degradation of ANFs such as chlorogenic acid, caffeic acid, phytic acid and saponins (Azza *et al.*, 2013; Hassaan *et al.,* 2018). In particular, *Bacillus subtilis* has been successfully used in SSF process to improve bio-availability of feed nutrients (Kiers *et al.,* 2003), to produce

biologically functional products used as enzyme and probiotics enriched immune regulators and performance promoters (Kumar and Duhan, 2011 and Yamamoto *et al.,* 2007), or to obtain new alternative feeding materials which were reduced in ANF and fibre contents (Nair, 1990; Pal vig and Walia, 2001 and Barnes *et al.,* 2012; Safari *et al.,* 2012; Nutraferma, 2014 and Zhang *et al.,* 2014).

The strain of *Bacillus subtilis ATCC PTA-6737* is a safe microorganism, and has been widely used in solid state fermentation processes for the production of feed additives, mainly probiotics and enzymes and enrichment of feed materials (Nutraferma, 2014 and Zhang *et al.*, 2014). Optimum conditions for the cultivation of *Bacillus subtilis ATCC PTA-6737* were 37 ^{0}C of temperature and 7.0 of pH under aerobic condition for 12 h using fermentation nutrient broth according to the culture collection centres of ATCC of the global bioresource centre and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). These conditions were optimised for its application in SSF processes, usually ranging from a 30-37 ^{0}C of temperature, 6.5-7.0 of pH, 40 to 80 % of moisture content of fermenting substrate, 20 to 150 rpm (round per min) of stirring, under aerobic condition of 0.25 to 0.75 L/min of aeration rate for 24 to 72 h using plant feed materials such as soybean meal, soybean, corn, cereal grains and some other agro-wastes such as fruit pomaces its application in SSF (Gessese and Mamo 1999; Joseph *et al.,* 2008; Chantet *et al.,* 2011; Azza *et al.,* 2013; Zhang *et al.,* 2014; Shi *et al.*, 2017).

Fermented SFM with nutritionally improved qualities could be an alternative feeding material in monogastric animal nutrition as opposed to their use in ruminants. A recent study showed that fermentation of SFM for the purpose of pig and poultry nutrition resulted in improved nutrient content (particularly increased protein and degraded crude fiber) and reduced dietary ANFs (Poulsen and Blaabjerg 2017). Nutritionally enriched SFM fermented with yeast and *Bacillus subtilis* in SSF process was even successfully incorporated up to 25% into a compound fish diet (Soltan *et al.,* 2015; Hassaan *et al.,* 2018). Moreover, the diets of shrimps could be added up to a 5% inclusion of fermented SFM with fungal microorganism (Jannathulla *et al.,* 2018).

All the above research studies indicated that SSF using safe microorganisms could lead to successful enrichment treatment of SFM which could have a high potential for feeding of monogastric animals. Moreover, SSF using bacteria inoculant such as *Bacillus subtilis* could be of significant importance to produce nutritionally enriched feed material. To our best knowledge, the effect of *Bacillus subtilis* fermentation under optimum fermentation condition on the nutritional composition of SFM has been rarely investigated.

Therefore, this study was conducted to ferment SFM with two moisture contents (66 and 80%, w/w) by *Bacillus subtilis ATCC PTA-6737* fermentation for nutritional enrichment.

MATERIALS AND METHODS

Bacillus subtilis ATCC PTA-6737 purchased from ATCC (LGC Standards GmbH Mercatorstr. 51 46485 Wesel Germany) in the form of lyophilised pellet was cultivated on Brain Heart Infusion Broth at 37 °C for 24 h to collect sufficiently enough inoculant for fermentation. The cultivated bacteria were immersed in saline solution used as inoculant and its bacteria count determined as cfu (colony forming unit) /g wet mass (w/w) by the method reported by ISO (2004) at 37°C for 24 h. SFM sample was bought from a local feed mill and ground to pass a 3-mm sieve. Autoclaved sample of SFM in glass flasks at 121°C for 60 min was added with sterilised distilled water to obtain a sample with 66 or 80% moisture content (w/w), followed by inoculation of bacteria at a rate of $1x10^7$ cfu per g solid material, and left for fermentation at 37 °C, pH=7.0, aeration rate of 0.75 L/min with a constant stirring of 150 rpm for 0, 24, 48 and 72 h using an orbital benchtop shaker (Thermo Scientific Inc.) with the control of fermentation parameters. These fermentation condition for SSF of SFM were carefully selected from the literature (Gessese and Mamo 1999; Joseph *et al.,* 2008; Chantet *et al.,* 2011; Azza *et al.,* 2013; Zhang *et al.,* 2014; Shi *et al.*, 2017). The experimental design was of 2 moisture levels (66 and 80%, w/w) x 3 replicates x 4 incubation periods (0, 24, 48 and 72 h). At the end of each incubation period, bacteria count (ISO 2004) and pH values of fermenting substrate were immediately measured. And then wet samples were dried at 50 °C until a constant weight of sample was obtained. The crude protein, crude fibre and crude ash were determined by the methods of AOAC (1990). The content of soluble nitrogen and organic acids (mainly

Table 1. Changes in pH, bacteria count (cfu/g of dried sample) and organic acids (% of dried sample) of fermented SFM

Time (h)	pH	Bacteria count cfu/g of dried sample	Acetic acid %	Butyric acid %	Lactic acid %
66% moisture content (w/w)					
0	5.73±0.11^{a}	3.3x10^{6}±0.05^{a}	2.20±0.33^{a}	1.15±0.05^{a}	0.95±0.05^{a}
24	7.67±0.15^{b}	2.8x10^{9}±0.03^{b}	4.74±1.73^{b}	0.00±0.00^{b}	5.5±1.42^{b}
48	9.1±0.13^{c}	4.1x10^{9}±0.015^{c}	1.08±0.022^{c}	0.54±0.30^{c}	3.18±1.29^{b}
72	8.67±0.17^{c}	5.9x10^{9}±0.010^{d}	1.47±0.42^{c}	0.06±0.03^{b}	1.09±0.14^{c}
80% moisture content (w/w)					
0	5.84±0.10^{a}	3.6x10^{6}±0.02^{a}	2.0±0.3^{a}	1.15±0.05^{a}	0.95±0.05^{a}
24	7.50±0.12^{b}	2.2x10^{9}±0.01^{b}	2.19±0.006^{a}	0.00±0.00^{b}	1.24±0.011^{b}
48	8.07±0.14^{c}	7.3x10^{9}±0.015^{c}	2.04±0.12^{a}	0.00±0.00^{b}	1.18±0.20^{b}
72	7.15±0.18^{b}	9.5x10^{9}±0.60^{e}	4.35±0.001^{b}	0.07±0.005^{b}	1.70±0.03^{c}

Note: Different letters in the same column indicated significant (Duncan test, *P<0.05*) differences between the means ± standard deviation.

acetate, lactate and butyrate) were determined by the methods reported by Karabulut and Canbolat (2005). Phytate content was determined spectrophotometrically by the method of Raheja *et al.* (1973). Protease activity (IU/g) was determined as ability to hydrolysis 0.8% (w/v) azocasein protein by the method of Cotta *et al.* (1986). For all microbiological and chemical analysis, 9 (3x3) measurements were performed per treatment (3 times of analysis of each of 3 samples (flasks) taken at the end of each fermentation period). The results of cfu and nutrients were expressed on the basis of dry matter of samples. .

A general linear model (GLM) was used to test the effect of water levels on the studied parameters using SPSS statistical program (IBM SPPS Inc, version 23), where the differences between the means of treatments were separated by Duncan' Multiple Comparison Test at a 0.05 significance level.

RESULTS AND DISCUSSIONS

The pH of SFM was significantly (*P<0.05*) influenced by the incubation time, but not by the water content of SFM (Tab. 1). Increasing the incubation time induced significant (*P<0.*05) increases in the pH from 5.73 at 0 h to 9.1 at 48 h of fermentation. As overall, the production of total organic acid did not greatly change during the fermentation of SFM between the SFM samples with 66 or 80% moisture contents (w/w), but there were sporadic changes in acetic and lactic acids over the fermentation period. Similarly, significant (*P<0.05*) increases in bacterial growth were observed with fermentation of SFM with both moisture levels. The changes in fermentation parameters in this study indicated successfully optimized fermentation. The range of pH values in our study was similar to earlier results reported by Sneath (1986), and the raised pH values in our study was found as a result of the production of alkaline protease by *Bacillus subtilis* (Sarkar *et al.,* 1993 and Chantawannakul *et al.,* 2002). If the desired aim is to produce more alkaline protease enzyme in SSF process we recommend that the fermentation period must be extended over a period of 72 h, similar to the our study. SFM was reported to be a good substrate of producing alkaline protease in SSF studies (Haq and Mukhtar, 2004). Alkaline protease production in our study (Tab. 2) was significantly increased (P<0.05), which was also associated with increased amount of soluble amino acid nitrogen in our study, and the effect of moisture content of SFM on these parameters was insignificant. Similar increases in soluble amino acid nitrogen were reported by Kiers *et al.* (2000). It was earlier shown that the production of alkaline protease can be stimulated at high pH condition above 6.0 with constant stirring (Abusham *et al.,* 2009), similar to the conditions set up in our study. The sporadic changes in individual organic acids seen in study

Table 2. Effect of fermenting SFM at two levels of moisture content on nutritional composition (analysed nutrients were expressed as % of dried sample)

Parameters %	Moisture % (w/w)	0 h	24 h	48 h	72 h
Dry matter %	66	34.0 ± 0.05^{aA}	30.11 ± 0.44^{bA}	28.18 ± 0.19^{cA}	29.4 ± 0.33^{cA}
	80	20.0 ± 0.01^{aB}	19.07 ± 0.07^{bB}	18.15 ± 0.04^{cB}	18.4 ± 0.29^{cB}
Crude ash %	66	6.55 ± 0.08^{aA}	7.14 ± 0.13^{bA}	9.77 ± 0.18^{cA}	10.0 ± 0.14^{cA}
	80	6.55 ± 0.08^{aA}	9.47 ± 0.18^{bB}	11.88 ± 0.19^{cB}	9.14 ± 0.23^{bB}
Crude protein %	66	35.25 ± 0.014^{aA}	35.11 ± 0.30^{aA}	47.06 ± 0.18^{bB}	47.87 ± 0.47^{bA}
	80	36.35 ± 0.10^{aA}	47.25 ± 0.77^{bB}	45.97 ± 0.18^{bB}	43.95 ± 0.76^{cB}
Crude fibre %	66	$39,21\pm0,2^{aA}$	$36,66\pm1,14^{bA}$	$32,79\pm0,74^{aA}$	$36,99\pm0,03^{bA}$
	80	$39,61\pm0,22^{aA}$	$37,07\pm1,37^{bA}$	$35,32\pm0,79^{bB}$	$34,68\pm0,55^{bB}$
SAN %	66	4.8 ± 0.44^{aA}	11.44 ± 0.25^{bB}	9.34 ± 0.03^{cA}	10.29 ± 0.03^{dA}
	80	4.8 ± 0.44^{aA}	11.80 ± 0.18^{bB}	11.88 ± 0.03^{bB}	11.70 ± 0.65^{bA}
Protease Activity IU/g	66	2.5 ± 0.4^{aA}	6.70 ± 0.05^{aA}	6.75 ± 0.01^{aA}	7.55 ± 0.01^{bA}
	80	2.5 ± 0.4^{aA}	7.31 ± 0.2^{bA}	7.40 ± 0.3^{cB}	8.70 ± 0.4^{dA}
Phytic acid %	66	0.40 ± 0.02^{aA}	0.29 ± 0.001^{bA}	0.23 ± 0.05^{bA}	0.23 ± 0.04^{bA}
	80	0.40 ± 0.02^{aA}	0.26 ± 0.02^{bA}	0.27 ± 0.01^{bA}	0.25 ± 0.01^{bA}

Note: Different letters in the same column indicated significant (Duncan test *P<0.05*) differences between the means ± standard deviation. Different letters in the same row indicated significant (Duncan test *P<0.05*) differences between the means ± standard deviation.

were found contradictory to the results of Ohara and Yahata (1996) obtained from the fermentation of many species of *Bacillus* at anaerobic conditions.

Fermentation SFM caused a significant reduction in dry matter contents of SFM with both moisture levels (*P<0.05*), while the effect of 80% water level was more pronounced (Tab. 2). In addition, there were significant increases (*P<0.05*) in crude ash contents of SFM with both levels of moisture. There were also significant increases (*P<0.05*) in crude protein contents of SFM with both levels of moisture. The increased crude protein content was higher (*P<0.05*) in SFM with 66% than 80% moisture content (w/w). Total increase in the level of crude protein was about 36% in this study (Tab. 2). The level of crude fibre was significantly (*P<0.05*) reduced about 18% in total, and the fermentation effect on reduced crude fibre was higher in SFM with 66% than 80% water content (w/w). Phytic acid considered as ANF in monogastric animals were remarkably degraded about 37-42% irrespective to the moisture contents of SFM. ANFs were earlier shown to be remarkably degraded in SSF studies (Azza *et al.*, 2013; Hassaan *et al.,* 2018). Previous studies using *Bacillus subtilis ATCC 6633*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus sp. AR-009* and *Bacillus subtilis natto* fermentations of various feed materials including SFM have reported a 3 to 104% increase of crude protein, a 17% reduction of crude fibre and a 80% increase of crude ash (Zhang *et al.,* 2014). The same results regarding enrichment of nutrients and reduction of its phytic acid content were also reported with *Bacillus subtilis* fermentation of SBM (Teng *et al.,* 2012; Dai *et al.,* 2017; Yuan *et al.,* 2017).

A recent study at our laboratory indicated that the same fermented SFM with ATCC PTA-6737 could not be fed more than 20% of the compound feed of young growing Carp (*Cyprinus carpio*) (Yigit *et al.,* 2017). This results was in good agreement with other results reported elsewhere (Soltan *et al.,* 2015; Hassaan *et al.,* 2018; Jannathulla *et al.,* 2018), where the SFM fermented with *Bacillus subtilis* can be fed to the fish and shrimps up to 25% and 5% of total diet, respectively.

According to the results fermenting SFM of high levels of moisture content (66 to 80%, w/w) with *Bacillus subtilis* produced a feed material containing reduced phytic acid and crude fibre which was associated with protein, ash and protease enrichment. This new feed material

can be used as alternative protein source or a functional feed material in farm animal nutrition.

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