



## Solid-State Fermentation of Corn and Soybean by *Bacillus subtilis* Egyptian Isolate

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### ABSTRACT

The major ingredients of poultry and animal diets are corn and soybean. In the current study, *Bacillus subtilis* isolate was used to ferment soybean and corn. Total phenolic content, antioxidant activity, antimicrobial activity and some nutrients of fermented soybean and corn have been investigated. Antioxidant activity and phenolic content of fermented corn and soybean significantly increased after fermentation of soybean while decreased in fermented corn. Fermented soybean and corn exhibited varying degrees of inhibitory activity against eight pathogenic microorganisms. The amount of crude protein and ash percentage of both fermented corn and soybean increased while carbohydrate and oil content decreased during fermentation of both substrates. As a consequence, fermented soybeans should be added to animal feed.

### INTRODUCTION

Solid-state fermentation is defined as process used to produce some metabolites generated by microorganisms grown on a solid such as soybean or wheat bran for industrial applications, that process consists of depositing a solid culture substrate such as soybean or wheat bran in a temperature-controlled room with low water level (Raimbault, 1980; Pandey, 2003). *Bacillus subtilis* is a Gram-positive bacterium, facultative anaerobe, endospore-forming rod and catalase positive. (Nakano and Zuber, 1998). This species is commonly found in the upper layers of the soil, in human gut and in higher elevation (Hong *et al.*, 2009, Sudhagar and Reddy, 2017). *B. subtilis* ferments traditional foods and changes nutritional properties of these foods (Ashok, *et al.*, 2010). Corn (*Zea mays*) and soybean (*Glycine max*) are the most commonly used in animal feed. Soybean is source of protein and energy of animal diet (Li *et al.*, 1990).

Previous studies have shown that the positive role of fermentation on the nutritional value of animal feed by increasing the bioavailability of nutrients. In addition, the growth performance of animals and the gastrointestinal health of animals (Song *et al.*, 2010). Plant wastes have a potential source of bioactive compounds and antioxidants, which could be used as functional ingredients in animal feeds (Fontana *et al.*, 2013). Antioxidants donate one of their electrons or hydrogen to free radicals, stopping their chain reaction (Kaur and Kapoor, 2001). Supplementation of animal feeds with phenolic compounds may have positive effects on animal gut health by improving

antimicrobial activity (Ignat *et al.*, 2011). The aim of this study is concerning with the impact of solid-state bacterial fermentation on components and bioactivities of corn and soybean.

## MATERIALS AND METHODS

### 1. Microorganism and Basal Substrate:

*Bacillus subtilis* isolate was obtained from Botany and Microbiology department, faculty of Science, AL-Azhar University, Assiut branch was stored at 4°C and sub-cultured at 30°C for 24 h periodically. Table .1 shows the confirmation of identified *B. subtilis* by morphological and physiological means according to Bergey *et al.*, (1984)

**Table 1.** Characteristics of *B. subtilis* isolate

Test	Result	Test	Result
Gram stain	+	Hydrogen sulfide production	-
Shape	Rod	Indole production	-
Endospore	+	Acid from mannitol	+
Motility	+	Nitrate reduction	+
Catalase	+	Oxidase	+
Citrate (simmons)	+	Phenylalanine deaminase	-
Acid from sucrose	+	Acid from lactose	-
Gas from glucose	+	Urea hydrolysis	-
Gelatin hydrolysis	+	Voges-proskauer	+
Acid from glucose	+		

+: positive -: negative

### 2. Inoculum Preparation:

Bacterial suspension (1ml) was prepared by inoculated 100 ml suspension of nutrient broth (5g/100ml) after sterilization at 121°C for 15 min. The cultures were shaken overnight at room temperature. From this 5 ml were used as starter in the following experimental work.

### 3. Preparation of Solid-State Fermentation:

Soybean and corn obtained from a local market were ground to pass a 3 mm sieve and addition of water before autoclaving 2% (v/w), *Bacillus subtilis* activated on nutrient broth for 16 h for solid-state fermentation (SSF) as described Limón *et al.*, (2015).

The slandered inoculum of *Bacillus subtilis* added to ground soybean and corn mixed carefully under sterile conditions and incubated at 37° C for 24 hrs. All experiments were performed in triplicate.

### 4. Determination of total Phenolic Content:

Samples were prepared and extracted by adding 25 ml of 50 % ethyl alcohol on 0.5gm of each sample (fermented and non-fermented), then agitation for two hours, followed by centrifugation at 3000 rpm for 20 min. Total phenolic compounds (TPC) were examined using modified Folin– Ciocalteu method (Jaramillo-flores *et al.*,2003). The modification was achieved by mixing 900 µL of 10 fold

Folin–Ciocalteu phenol reagent (diluted 1:10 with distilled water) into 100  $\mu\text{L}$  aliquot of ethanolic extract and allowed to stand for 5 min at room temperature; then 0.75  $\mu\text{L}$  of 7% sodium bicarbonate solution was added to the mixture, vortexed for 30 s, and allowed to stand for 90 min at room temperature. The absorbance of the prepared samples was checked at 725 nm using a spectrophotometer (6505 UV/Vis, Jenway LTD., Felsted, Dunmow, UK). A calibration curve of Gallic acid (ranging from 0 to 1.00 mg/mL) was prepared and tested. All values were expressed as mean (mg Gallic acid equivalents/g of Dry weight for 3 replications).

#### 5. Determination of Antioxidant Activity:

Samples were extracted using methods described by (Zielinski *et al.*, 2008). The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay was carried out according to the method described by Lee *et al.*, (2003), with some modifications. The stock reagent solution (10-3Mol) was prepared by dissolving 22 mg of (DPPH) in 50 ml of methanol and stored at 20°C until use. The working solution ( $6 \times 10^{-5}$ Mol) was prepared by mixing 6 mL of stock solution with 100 mL of methanol to obtain an absorbance value of  $0.8 \pm 0.02$  at 515 nm, as measured using spectrophotometer. A 0.1 ml of extracted samples mixed with 3.9 ml of DPPH solution. Vortex for 30 s and left to react for 30 min, the absorbance was measured at 515 nm. The control without addition of extract was also analyzed. Scavenging activity was calculated as follows: DPPH radical scavenging activity (%) =  $[(\text{Ab control} - \text{Ab sample}) / \text{Ab control}] \times 100$  Where Ab is the absorbance at 515 nm.

#### 6. Determination of Antimicrobial Activity:

All samples were minced and extracted with 10 times methanol (1:10) (w/v) at room temperature for 5 h (repeated three times) and then filtered through Whatman No. 4 filter paper. The methanol extracts of each sample were concentrated at 40 °C under vacuum and

freeze-dried. Antimicrobial activity was evaluated against eight microorganisms (source: Department of Microbiology and Immunology, Faculty of Veterinary, Sohag University, Egypt) using agar well diffusion assay (Schillinger, 1989).

#### 7. Determination of Chemical Composition of Fermented and Non-Fermented Samples:

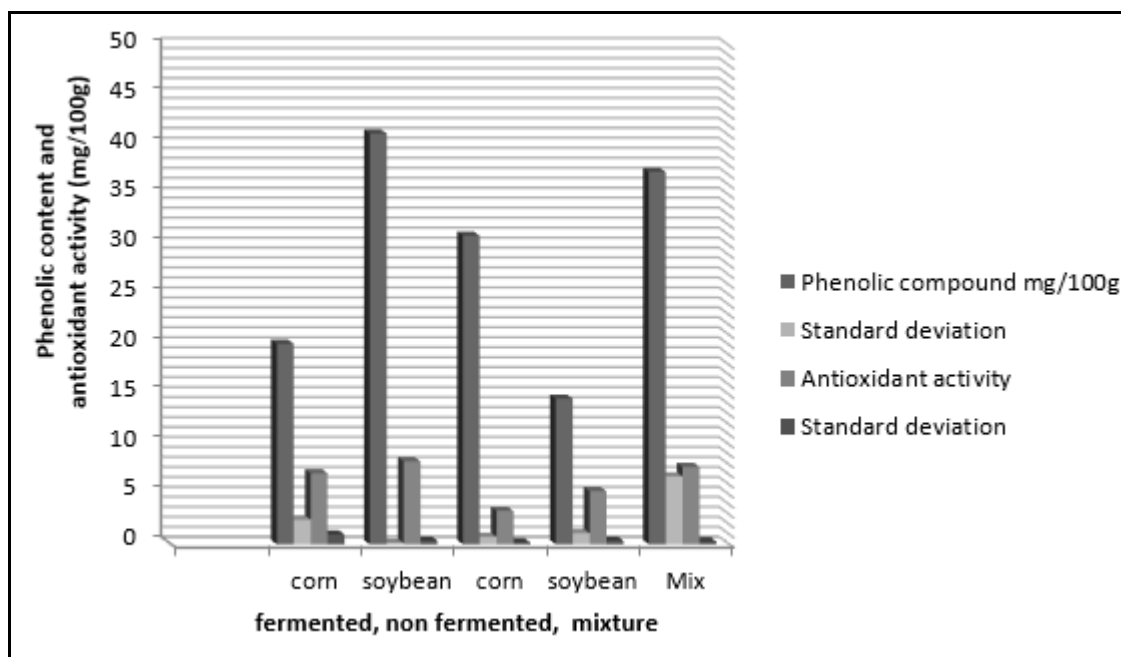
Moisture, protein, fat and ash contents were determined according to official methods as described in A.O.A.C. (2000). Carbohydrate content estimation by Phenol sulphuric acid method (Masuko *et al.*, 2005).

#### 8. Statistical Analysis:

The studied data analyzed by using SAS program (SAS Institute 2008. v9.2, USA). Comparison of total mean value of each trait done by used the revised LSD (Petersen, 1985). Pearson correlation coefficient was calculated among studied data.

### RESULTS AND DISCUSSION

The data illustrated in figure 1 revealed that concentration of the phenolic compound in fermented soybean was 41.17 mg/100 g with standard deviation 0.12, fermentation of soybean and corn mixture was 37.33 mg/100 g with standard deviation 6.80 while fermentation of corn was 20.10 mg/100 g. The results showed that fermented soybean or corn alone generates much more phenolic content than the mixture. On the other hand, the antioxidant activity of soybean exhibited 8.32 mg/100 g, fermentation of mixture was 7.75 mg/100 g, while fermentation of corn was 7.12 mg/100 g, and the results showed that the fermentation of soybean or corn separately was much better than fermentation together. Total phenolic content increased in soybean after 72 h fermentation, it also activates antioxidants in comparison with the non-fermented soybean (Gracia, *et al.*, 2004). Our results are coinciding with data obtained by Robbins, 2003, who stated that total phenolic content increased in fermented soybean.



**Fig. 1** Phenolic content and antioxidant activity of unfermented and fermented soybean and corn.

Other results showed that fermented soybean was superior to soybean in most antioxidant properties (Yang et al., 2000). The fermentation play an important role in the improvement of antioxidation (Ga and Bae, 2011). Total phenolic and flavonoids contents in soybean supporting potential health benefit of animal feed . In addition, soybean fermentation could enhance the amount of these components (Samruan et al., 2014). It was observed that phenolic compound decreasing during natural lactic acid fermentation of maize (Awada et al., 2005). The increase in total phenolic content found in the fermented samples could be due to the bound phenolic being liberated during fermentation (Bartolome and Gomez-Cordoves, 1999). The decrease in phenolic compounds during fermentation could also be due to the acidic environment and the reduced extractability of the phenolic compounds (Towo, et al., 2006).

Table of 2 shows that the antimicrobial activity of soybean, corn and mix fermentation by *Bacillus subtilis*. Antimicrobial activity of fermented soybean and corn against eight selected microorganisms exhibited high activity by fermented soybean and corn-soybean

mixture while fermented corn was fewer antimicrobial activities. Antimicrobial activities of both fermented soybean and soybean-corn mixture against all tested microorganisms, showed that zones of inhibition ranged from 13 to 22 mm. fermented corn exhibited inhibition against *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Fusarium lateritium* while no inhibition against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Bacillus cereus* by fermented corn.

Antimicrobial properties of fermented mixture (soybean and corn) exhibited. Highest activity with inhibition zone 22 mm for *Fusarium lateritium*, *Candida albicans*, and 20mm inhibition zone against *Saccharomyces cerviaceae*, *Pseudomonas aeruginosa* and *Bacillus cereus*, 18 mm inhibition zone against *Staphylococcus aureus*, *Klebsiella pneumoniae* and 16ml inhibition zone against *Proteus vulgaris*. Antimicrobial activity of soybean showed that 22mm inhibition zone against *Fusarium lateritium*, 20mm inhibition zone against both *Candida albicans* and *Bacillus cereus* 19mm inhibition zone against *Saccharomyces cerviaceae*, 17mm inhibition zone against *Staphylococcus aureus*, 15mm

inhibition zone against *Proteus vulgaris*, 14mm inhibition zone against *Pseudomonas aeruginosa*, 13mm inhibition zone against *Klebsiella pneumoniae*, while antimicrobial activity of corn was 17mm inhibition zone against *Pseudomonas auroginosa*, 12mm

inhibition zone against *Candida albicans*, *Fusarium lateritium*, 11mm inhibition zone against *Staphylococcus cerviacea*. It has been reported that increased antimicrobial activity by fermentation of soybean (Fuller, 1989).

**Table 2.** Zone of inhibition (zone diameter- mm) of fermented corn and soybean extracts against pathogenic microorganisms.

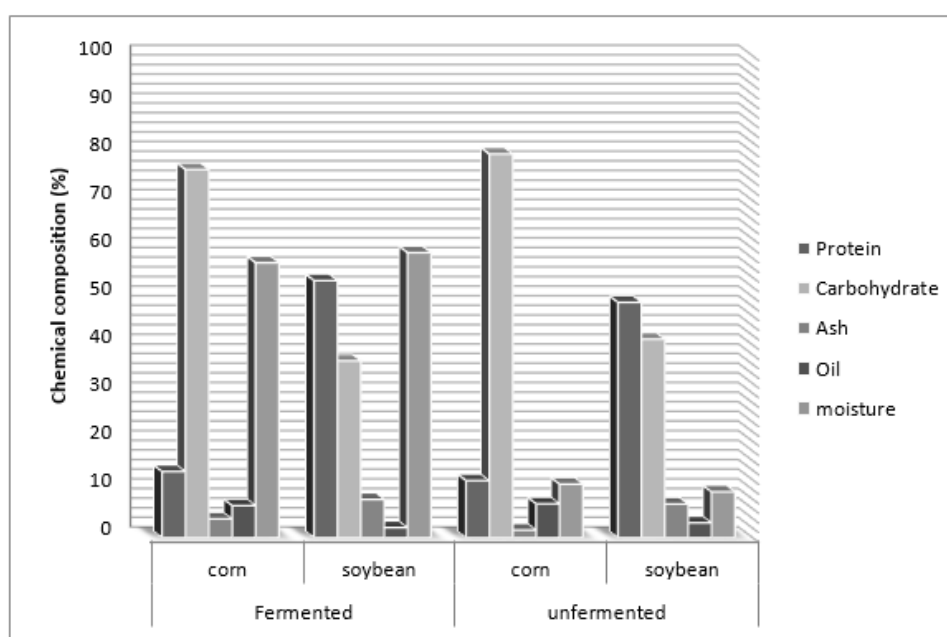
Microorganisms	Zone of inhibition (mm)		
	CSF	SSF	CSSF
<i>Staphylococcus aureus</i>	0	17	18
<i>Klebsiella pneumoniae</i>	0	13	18
<i>Proteus vulgaris</i>	0	15	16
<i>Bacillus cereus</i>	0	20	20
<i>Pseudomonas aeruginosa</i>	17	14	20
<i>Saccharomyces cerevisiae</i>	11	19	20
<i>Candida albicans</i>	12	20	22
<i>Fusarium lateritium</i>	12	22	22

CSF: Corn Solid-State Fermentation, SSF: Soybean Solid-State Fermentation, CSSF: Corn-Soybean mix Solid-State Fermentation

**Figure 2.** Shows that soybean total protein increased after fermentation (53.34) comparing with non-fermentable soybean (48.81), also, total protein of fermented corn was increased (13.07) comparing with non-fermentable corn (11.82), whereas total protein of fermented soy-corn mixture was (38.61). This result indicated that the fermentation of each substrate either soybean or corn separately better than fermentation within the mixture. Other studies have shown that soy antigenic proteins could be degraded during fermentation (Chi and Cho, 2016; Frias, 2008). Rozan *et al.*, 1996 showed that at the first stage of fermentation with *B.subtilis* effect on the characteristics of proteins in corn-soybean meal mixed feed.

Our result showed that the amount of protein and ash % after fermentation of corn

and soybean increased by 1.88, 4.53, and 1.57, 0.96 respectively (figure 2), whereas carbohydrates and oil decreased by 3.2, 4.51 and 0.73, 0.99 respectively. Other research results showed that there is significant increase of protein during fermentation of maize (43.5%), and total phenolic content (23.4%) (Cui *et al.*, 2012). Fermentation of soybean by *Bacillus natto*, increase of protein content with the highest content in the moisture. On the other hand contents of carbohydrates increased and ranged from 14.77 to 29.08 while those of crude fiber, fat and ash were generally low (Ojokoh and Wei, 2011). Decreasing of carbohydrate and oil contents may be due to that *Bacillus subtilis* utilized carbohydrate and oil during fermentation as the major source of energy (Yamabe *et al.*, 2007).



**Fig.2:** Effect of fermentation on chemical composition of corn and soybean.

Enhancement of antimicrobial and antioxidant activities of substrate either soybean or corn by fermentation may be releasing of bioactive peptides (Singh *et al.*, 2015) or due to generating of bioactive peptides during soybean fermentation (Kwon *et al.*, 2002 ). In addition to many oligopeptides produced from soy protein by microbial proteinase during fermentation, demonstrated a range of biological activities (Gibbs *et al.*, 2004).

### CONCLUSION

From our results, we can conclude that fermentation of both soybean and corn produce significant increase of protein content, antioxidant activity, and antimicrobial activities but negative influence on carbohydrate and oil contents. Therefore, this study recommended that fermented soybeans could be adding to animal feed.

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