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## **Effect of Temperature on Biochemical Changes Induced by *Bacillus subtilis* (SDA3) During Starter Culture Fermentation of Soybean into Condiment (Soy-Daddawa)**

B.O. Omafuvbe

Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria

**Abstract:** In an attempt to upgrade the traditional fermentation technology of soybean into daddawa, the effect of fermentation temperature on the biochemical and organoleptic properties of soy-daddawa produced by starter culture was studied. *Bacillus subtilis* SDA3 previously selected as a good starter for soy-daddawa production was used to ferment sterile dehulled cooked soybeans at 25, 30, 35 and 40°C for 72 h. The viable cell counts of *B. subtilis* SDA3 increased throughout the 72 h fermentation process at 25 to 35°C while the counts decreased after the 24th h at 40°C fermentation. pH value increased throughout the fermentation with a rather low increase in the fermentation at 25°C. Relative proteolytic activity increased with fermentation, attained a peak at 48 h and then dropped in fermentations at 30-40°C. Proteolytic activity which was not detected by the 12th h increased thereafter till the end of the fermentation at 25°C. Free amino acid content increased throughout the 72 h fermentation at 30-40°C while an initial drop was observed in the first 12 h with subsequent increase till the end of the fermentation at 25°C. Alpha amylase activity increased, attained a peak at the 48 h and then dropped in 30 and 35°C fermentations. Alpha amylase activity increased throughout the 72 h fermentation at 25°C while at 40°C, the activity attained a peak at the 24th h and then dropped. Fermentation at 35°C gave the highest levels of proteolytic and alpha amylase activities, pH and free amino acids in soybean inoculated with *B. subtilis* SDA3. Organoleptically, soybean fermented by *B. subtilis* SDA3 at 35°C produced the best quality soy-daddawa as judged by a panel of regular soy-daddawa consumers. Fermentation at 35°C was therefore chosen as the optimised temperature for the production of soy-daddawa by *B. subtilis* SDA3 starter culture.

**Key words:** Soybean, daddawa, *Bacillus subtilis*, temperature, fermentation

### **INTRODUCTION**

Soybean (*Glycine max*) is fermented locally by the majority of people in Benue and Plateau States of Nigeria into a flavoursome alkaline food called soy-daddawa (Omafuvbe *et al.*, 2000; Dike and Odunfa, 2003). The procedure for the production of traditional soy-daddawa has been fully described (Ogbadu and Okagbue, 1988a; Omafuvbe *et al.*, 2000). *Bacillus* species especially *Bacillus subtilis* have been identified as been predominant in the fermentation process and capable of producing organoleptically acceptable product as is the case with other alkaline fermented legume products (Ohta, 1986; Ogbadu and Okagbue, 1988a, b; Sarkar *et al.*, 1993, 1994; Omafuvbe *et al.*, 2002; Azokpota *et al.*, 2006). Information on the biochemical changes associated with the traditional fermentation of soybean into daddawa is well documented (Popoola and Akueshi, 1986; Omafuvbe *et al.*, 2000; Omafuvbe and Awowole, 2003; Dike and Odunfa, 2003). The organoleptic properties of soy-daddawa and the popular West African locust bean (*Parkia biglobosa*) daddawa (iru) are not significantly different (Omafuvbe *et al.*, 2002). The awareness of the food values of soybean has increased its use for daddawa production and incorporation in other food recipes in many house holds in Nigeria. With this recent development, the need for the optimization of the process conditions

for producing daddawa is necessary to guarantee improved and consistent quality which would in turn increase general acceptability of the product. In line with the optimization of the fermentation process, reports are available on the influence of added salt in the production of soy-daddawa (Omafuvbe, 1994), effect of dehulling methods of soybean on soy-daddawa production (Dakwa *et al.*, 2005; Omafuvbe *et al.*, 2007) and the use of *Bacillus* starter to ferment cooked sterile soybean (Omafuvbe *et al.*, 2002; Ikpeme *et al.*, 2003; Terlabie *et al.*, 2006).

The traditional fermentation of oil seeds into condiment has been reported to occur at a temperature range of between 28-42°C in Nigeria (Achi, 2005). Also the traditional fermentation of kinema (a soybean fermented food in India) has been reported to occur at a temperature range of between 25-35°C (Sarkar and Tamang, 1994). The varying temperature and microbial profile involve in the conventional natural fermentation of oil seeds into daddawa and similar products results in variations in quality from one batch to another. The aim of this study was to investigate the influence of temperature on the biochemical changes induced by *Bacillus subtilis* SDA3 (previously isolated from traditional soy-daddawa (Omafuvbe *et al.*, 2000)) during starter culture fermentation of dehulled cooked sterile soybean into daddawa.

## MATERIALS AND METHODS

This study was conducted in the Food Microbiology Laboratory, Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria between February 2006 and January 2007.

### Organism

*Bacillus subtilis* SDA3 strain previously isolated from natural fermenting soybean and reported to produce organoleptically acceptable soy-daddawa (Omafuvbe *et al.*, 2000, 2002) was used. The organism was maintained on nutrient agar (NA, Oxoid CM3) slopes in the refrigerator.

### Aseptic Preparation of Soy-Daddawa

Soybeans (*Glycine max*) approximately 2 kg were sorted to remove debris, roasted and dehulled as previously described (Omafuvbe *et al.*, 2007). The roasted dehulled cotyledons were washed with warm water (60°C) and dispensed in 50 g (wet wt.) amounts in several 250 mL conical flask plugged with cotton wool. The contents of the flasks were autoclaved at 121°C for 20 min to obtain dehulled cooked sterile beans.

Suspension of predominantly vegetative cells of *B. subtilis* SDA3 was prepared in maximum recovery diluent (MRD, Oxoid CM733) as previously described (Omafuvbe, 2006). The dehulled cooked sterile soybean held in 250 mL flasks were inoculated with 500 µL of the *B. subtilis* cell suspension (this gave approximately 10<sup>4</sup> cells g<sup>-1</sup> wet wt. of soybeans). The flasks were divided into 4 batches consisting of 12 flasks each. Each batch of flask was incubated at 25, 30, 35, 40±2°C for up to 72 h for their contents to ferment. Duplicate flasks were removed from each group of temperature at selected time interval for analysis.

### Viable Cell Count

Fermenting beans (5.0 g wet wt.) were homogenised with 45 mL of sterile MRD in a stomacher (Colworth Stomacher 400) for 2 min. Further dilutions were made in sterile MRD and 1.0 mL of appropriate dilutions was plated in duplicate in NA using the pour plate technique. Inoculated plates were incubated at 30°C for 48 h following which the colony were counted and expressed as log<sub>10</sub> colony forming units (cfu) g<sup>-1</sup> wet wt. of sample.

### pH Value

Fermenting beans (4.0 g wet wt.) were blended with 16 mL of distilled water in a homogeniser (Ika-Werk, Ultra Turrax T25, dispersing tool S25 GM at speed 2) for 2 min. The pH of the slurry was measured with a pH meter (Hanna Instruments 8520).

### **Reducing Sugar and Free Amino Acids**

The reducing sugars and free amino acids in the fermenting beans were extracted with 80% ethanol (v/v) as previously described (Omafuvbe *et al.*, 2000). The ethanolic extracts were appropriately diluted for the various determinations. The free amino acid content was estimated following the ninhydrin colorimetric method of Rosen (1957) using glycine as standard solution. Reducing sugar was estimated by the colorimetric method (Somogyi, 1945) using glucose as standard solution.

### **$\alpha$ -Amylase (EC 3.2.1.1) and Proteolytic (EC 3.4.24.4) Activities**

$\alpha$ -amylase enzyme in the fermenting beans was extracted with 0.1 M phosphate buffer (pH 6.0) as previously described (Omafuvbe *et al.*, 2000). The blue value assay method of Fuwa (1954) was adopted for the determination of  $\alpha$ -amylase activity. One unit of activity was defined as the amount of enzyme that produced a 10% reduction in the intensity of blue colour in 30 min under the experimental conditions.

Proteolytic enzyme in the fermenting soybeans (3.0 g wet wt.) was extracted with 0.05 M phosphate buffer (pH 7.0) as previously described (Omafuvbe, 2006). The assay procedure was based on the method described by Sarkar *et al.* (1994) using azocasein (2.5 g L<sup>-1</sup>; Sigma A2765) as substrate. One unit of proteolytic activity is defined as the amount that produced an absorbance increase of 0.01 units in 30 min under the assay conditions.

### **Sensory Evaluation**

The sensory attributes of the soy-daddawa samples fermented at different temperature by *Bacillus subtilis* SDA3 starter were evaluated for preference by a panel of 10 regular daddawa consumers. The freshly fermented soy-daddawa samples were coded, randomly presented in saucers and assessed for colour, odour, taste and texture using a 100-point score card designed for the purpose. The data obtained were subjected to one way analysis of variance (ANOVA) followed by Student-Newman-Keul post hoc test (Primer for Biostatistics software package version 3.01 (Glantz, 1992). Statistical significance was accepted at p-value equal to or less than 0.05.

## **RESULTS AND DISCUSSION**

The changes in viable cell counts of *Bacillus subtilis* SDA3 during starter culture fermentation of soybean at different incubation temperatures (25, 30, 35 and 40°C) into soy-daddawa is shown in Fig. 1. *Bacillus subtilis* SDA3 grew well at the temperature range (25-40°C) studied. This indicated the mesophilic nature of *Bacillus subtilis* SDA3. The viable cell counts increased sharply in the first 12 h of fermentation of soybean at 30-40°C while the increase was rather low at 25°C. The growth rate of *Bacillus subtilis* SDA3 was faster at 30 and 35°C throughout the 72 h fermentation period. However, the viable cell counts increased during fermentation at 25, 30 and 35°C till the end of fermentation while the counts decreased after the 24 h of fermentation at 40°C. The pattern of growth of *B. subtilis* SDA3 observed during fermentation at 35 and 40°C is in support of the reports of Tamang and Nikkuni (1998) on the growth of *B. subtilis* KK2:B10 during kinema fermentation at these temperatures.

The pH value of the fermenting soybeans increased with fermentation time at the various incubation temperatures (Fig. 2). The pH rose from 6.51-7.68, 6.51-8.29, 6.51-8.40 and 6.51-8.07 at 25, 30, 35 and 40°C incubation temperatures, respectively. Fermentation at 25°C gave the least increase while fermentation at 35°C had the highest increase in pH. Increase in pH is a common feature in the fermentation of vegetable proteins (Steinkraus, 1996).

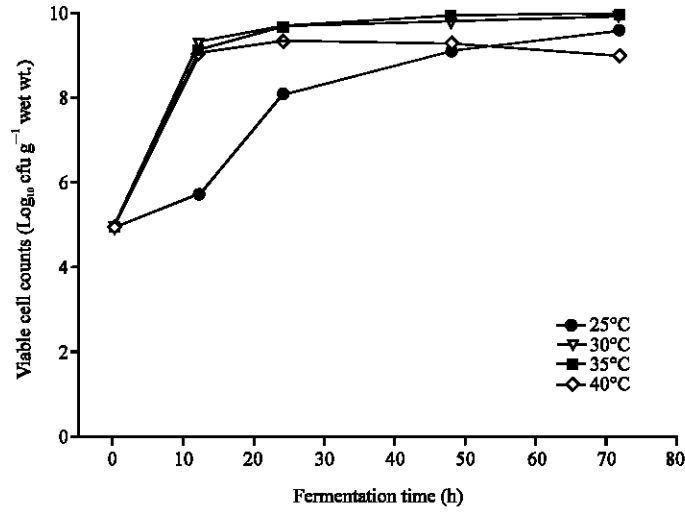


Fig. 1: Changes in viable cell counts of *Bacillus subtilis* SDA3 during soy-daddawa fermentation at different incubation temperatures. Values are means of determinations on duplicate fermentations

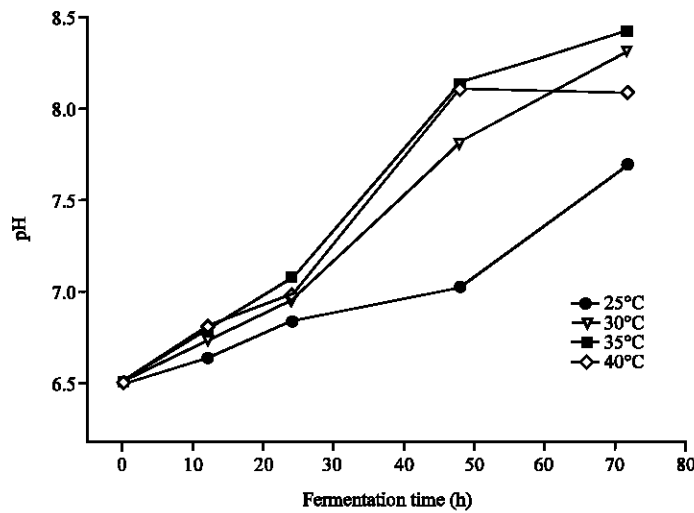


Fig. 2: Changes in pH during soy-daddawa fermentation by *Bacillus subtilis* SDA3 at different incubation temperatures. Values are means of determinations on duplicate fermentations

The relative proteolytic activity in the fermenting soybean increased rapidly, attained a peak by the 48th h of fermentation at 30-40°C and then dropped (Fig. 3). On the other hand, there was no proteolytic activity by the 12 h of fermentation in soybean fermented at 25°C. This may be related to the rather very low increase in the viable cell counts observed at 25°C during this period (Fig. 1). However, activity was detected by the 24 h of fermentation and this increased up till the 72 h of fermentation (Fig. 3). The period of rapid increase in relative proteolytic activity at the different fermentation temperatures coincides with the period of rapid increase in viable cell counts. It is significant to note that inoculated soybean incubated at 35°C exhibited a larger increase in proteolytic activity throughout the fermentation period.

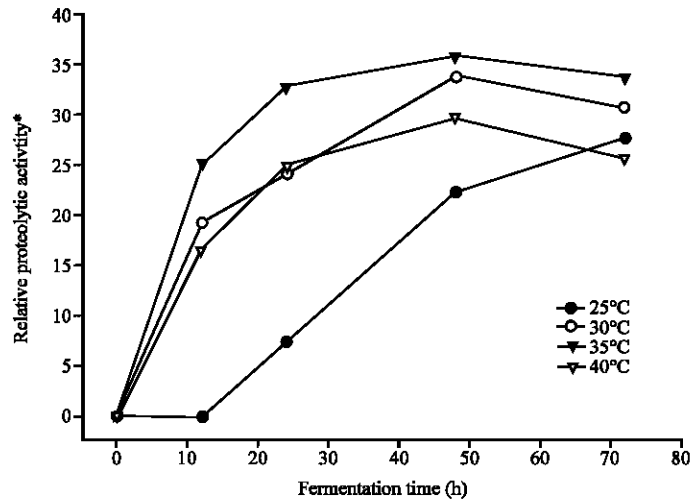


Fig. 3: Relative proteolytic activity during soy-daddawa fermentation by *Bacillus subtilis* SDA3 at different incubation temperature. \*Measured as activity on azocasein. Values are means of determinations on duplicate fermentations

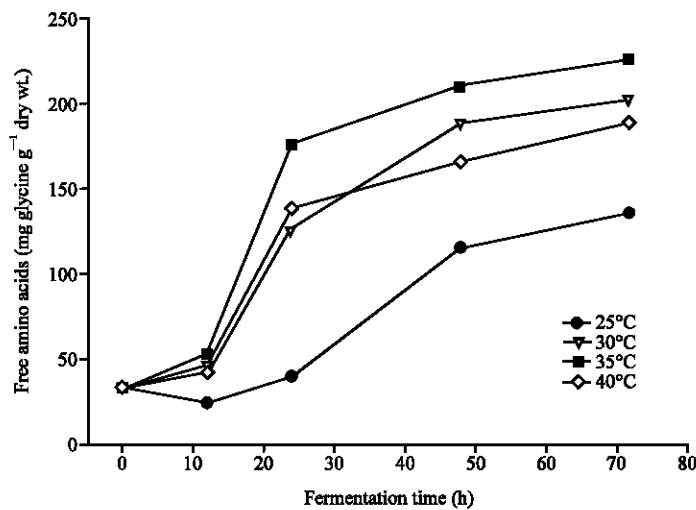


Fig. 4: Changes in free amino acids during soy-daddawa fermentation by *Bacillus subtilis* SDA3 at different incubation temperatures. Values are means of determinations on duplicate fermentations

Free amino acids increased rapidly during the fermentation of soybean inoculated with *Bacillus subtilis* SDA3 incubated at different temperatures (Fig. 4). The level of proteolytic activity exhibited at the various incubation temperatures coincides with the level of free amino acids produced. The free amino acids dropped in the first 12 h and then increased subsequently in soybean fermented at 25°C. Fermentation at 25°C exhibited the least level of increase while fermentation at 35°C exhibited the highest level of increase in free amino acids during the fermentation process. Protein hydrolysis has been reported as the most significant biochemical change occurring during soy-daddawa fermentation

(Omafuvbe *et al.*, 2000; Dike and Odunfa, 2003; Terlabie *et al.*, 2006). This is due to the rapid production of amino acids resulting from high proteolytic activity of the fermenting organisms.

Alpha amylase activity increased rapidly in the first 24 h of fermentation and dropped thereafter in soy-daddawa fermented at 40°C (Fig. 5). In soybean fermented at 30 and 35°C,  $\alpha$ -amylase activity increased, reached a peak at 48 h and dropped slightly by the 72 h of fermentation. Alpha amylase activity in fermentation at 25°C increased throughout the fermentation process and exhibited the least level of increase in activity. It is significant to note that fermentation at 40°C exhibited the highest level of activity in the first 24 h of fermentation (Fig. 5). After the 24 h of fermentation however,  $\alpha$ -amylase activity was higher in the fermentations at 30 and 35°C, respectively.

The level of reducing sugars in the fermenting soybean showed similar pattern of change in all the fermentations (Fig. 6). There was a general increase in the level of reducing sugars in the first 24 h followed by a decrease during soy-daddawa production at the various fermentation temperatures. Soy-daddawa fermented at 35°C gave the highest level of reducing sugar at the end of the fermentation process. It is significant to note that the period of rapid increase in the level of reducing sugars coincides with the periods of increase in  $\alpha$ -amylase activity (Fig. 5) and the growth rate of *B. subtilis* SDA3 as shown in the viable cell counts (Fig. 1) during the fermentation process. This is an indication that the reducing sugars of the fermenting soybeans were used by the fermenting organism for its metabolic activities.

The organoleptic attributes of soy-daddawa produced by *B. subtilis* SDA3 starter culture fermentation at 25°C were scored low and the scores were significantly different ( $p < 0.05$ ) from soy-daddawa produced at 30 and 35°C (Table 1). Soy-daddawa produced at 30 and 35°C were significantly different ( $p < 0.05$ ) in their scores for aroma and taste attributes while there was no significant difference ( $p > 0.05$ ) in colour, texture and total scores. Also, soy-daddawa produced at 35°C was significantly different from soy-daddawa produced at 40°C in all the organoleptic attributes scored (Table 1). It is important to note that the panellists showed preference for soy-daddawa produced at 35°C in all the organoleptic attributes scored. The organoleptic superiority of soy-daddawa produced at 35°C over others is presumably associated with the observed increased levels of biochemical activities during the fermentation and soluble products (especially free amino acids).

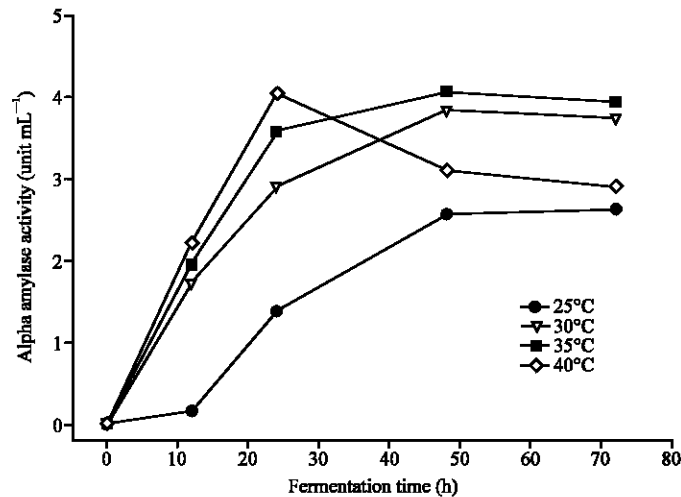


Fig. 5: Alpha amylase activity during soy-daddawa fermentation by *Bacillus subtilis* SDA3 at different incubation temperatures. Values are means of determinations on duplicate fermentations

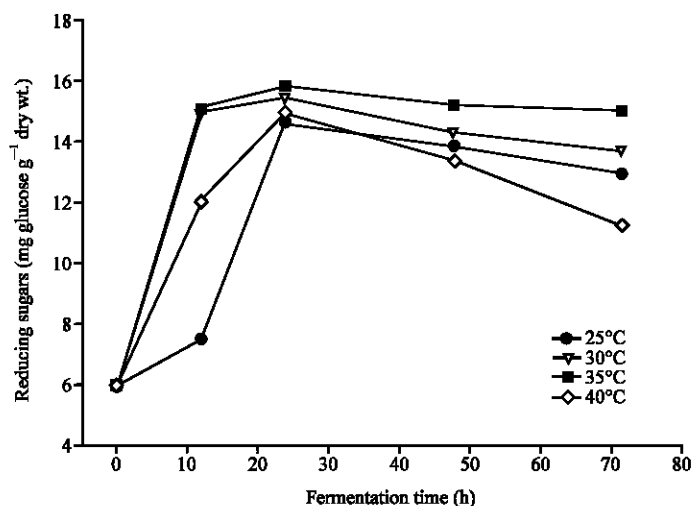


Fig. 6: Changes in reducing sugars during soy-daddawa fermentation by *Bacillus subtilis* SDA3 at different fermentation temperatures. Values are means of determinations on duplicate fermentations

Table 1: Average sensory evaluation of soy-daddawa produced by *Bacillus subtilis* SDA3 fermentation of soybean at different incubation temperatures

Organoleptic attributes	Temperature of incubation (°C)			
	25	30	35	40
Color (5)	2.0±0.3 <sup>a</sup>	3.0±0.2 <sup>bd</sup>	4.0±0.3 <sup>bc</sup>	3.5±0.5 <sup>cd</sup>
Aroma (30)	15.8±0.2 <sup>a</sup>	22.4±1.2 <sup>d</sup>	25.9±0.9 <sup>b</sup>	20.4±0.6 <sup>cd</sup>
Taste (40)	20.0±2.0 <sup>a</sup>	30.0±1.0 <sup>f</sup>	34.7±0.8 <sup>b</sup>	28.2±1.0 <sup>cd</sup>
Texture (25)	15.0±1.0 <sup>a</sup>	20.0±1.0 <sup>bc</sup>	22.0±0.8 <sup>b</sup>	18.0±0.7 <sup>c</sup>
Total score (100)	52.8±3.5 <sup>a</sup>	75.4±3.4 <sup>bd</sup>	86.6±6.2 <sup>bc</sup>	70.1±2.8 <sup>d</sup>

Data represent the mean scores±SE (n = 10). Means bearing different superscripts within each row differ significantly (p<0.05)

This is in support of previous report on the optimised temperature (35°C) for the production of African locust bean iru (Odunfa and Adewuyi, 1985). However, in other investigations, 37°C was reported to improve the organoleptic quality of African locust bean daddawa (Ikenebomeh, 1989), fermented cotton seed condiment (Sanni *et al.*, 1998) and kinema (Sarkar and Tamang, 1994).

The data obtained in this study suggest 35°C as the optimized temperature for the fermentation of dehulled cooked sterile soybean by *Bacillus subtilis* SDA3 starter culture into soy-daddawa.

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