# BIOCHEMICAL CHARACTERIZATION OF MULTIPLE STARTER CULTURES FERMENTATION OF SOYBEAN (*GLYCINE MAX* L.) SEEDS FOR SOYBEAN DADDAWA PRODUCTION

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***Abstract***
*Previous optimization attempts of soybean daddawa production based on the use of only Bacillus starters did not perfectly replicate the desirable qualities of spontaneous fermentation. However, recent optimization study on soybean daddawa production based on the use of multiple starter cultures (Bacillus subtilis LB3, Staphylococcus xylosus SAU3 and Leuconostoc mesenteroides ssp cremoris LAB5) appeared to hold great promise for soybean daddawa industrialization. In the present report, the biochemical characterization of such multiple starter cultures fermentation is presented. Seeds of soybean, Glycine max (L) Merr, were fermented into daddawa using the three microorganisms both singly and in combinations, with spontaneous fermentation serving as control set-up. The different fermentation sets were assessed for protease and α-amylase enzyme activities (U/ml), Free Amino Acids (FAA) and Total Soluble Sugars (TSS) (mg/g) as fermentation progressed. Changes in pH, Titratable acidity (TTA) (% lactic acid equivalent), Water Absorption Capacity (WAC) and Fat Absorption Capacity (FAC) (% ) were also monitored with fermentation time. Both protease and α-amylase enzyme activities as well as FAA and TSS of the fermenting daddawa increased with fermentation time. The use of multiple starter cultures significantly ($P<0.05$) improved protease activity. Recorded pH and TTA values increased in fermenting daddawa from 5.22 to 8.84 and 0.006 to 0.041 respectively. The WAC values increased from 210 to 390 with fermentation while FAC values were not affected by fermentation. Results of the present optimization studies hold great promise for daddawa industrialization.*

**Key words**: Industrialization; Optimization; Starter Culture; Soybean daddawa.

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## INTRODUCTION

Soybean daddawa is a product of spontaneous fermentation of soybean (*Glycine max* L.) seeds. It is used as soup condiment in many West African countries (Popoola and Akueshi, 1985; Omafuvbe et al., 2000). In addition to its flavor enhancing property, soybean-daddawa is also a good source of dietary protein and minerals (Popoola and Akueshi, 1986).

Studies have shown that, in addition to *Bacillus*; *Micrococcus* spp., *Staphylococcus* spp, *Leuconostoc mesenteroides* and *L. dextranicus* have been reported to be involved in soybean-daddawa fermentation (Antai and Ibrahim, 1986; Ogbadu and Okagbue, 1988; Dakwa et al., 2005; Edema and Fawole, 2006). However, previous optimization studies have reported on the use of single cultures of *Bacillus subtilis*, *B. licheniformis, B. pumilus* or in combinations (Suberu and Akinyanju, 1996; Omafuvbe et al., 2002). Recently, Afolabi and Abdulkadir (2016) improved on the restrictive use of *Bacillus* starter by incorporating *Leuconostoc mesenteroides* to *Bacillus subtilis* fermentation of soybean-daddawa. It is noteworthy that these optimization attempts achieved good quality end products, but the traditional aroma of soybean daddawa was not perfectly replicated.

Meanwhile, Achi (2005), had opined that the use of mixture of microorganisms with complimentary physiological and metabolic properties seems to be the best approach for

obtaining a condiment product with the desired nutritional and sensory properties. In this regard, a very recent report has documented the use of *Bacillus subtilis* LB3, *Staphylococcus xylosus* SAU3 and *Leuconostoc mesenteroides* ssp *cremoris* LAB5 for controlled fermentation of soybean-daddawa, in which the naturally fermented sensory attributes of soybean daddawa was nearly replicated in a controlled setting (Kolapo et al., 2019). In the present study, we report on the biochemical characterization of such multiple starter cultures fermentation of soybean-daddawa using the trios of *Bacillus subtilis* LB3, *Staphylococcus xylosus* SAU3 and *Leuconostoc mesenteroides* ssp *cremoris* LAB5.

## MATERIALS AND METHODS

### Starter Cultures

Previously typed, screened and selected cultures of *Bacillus subtilis* LB3, *Staphylococcus xylosus* SAU3 and *Leuconostoc mesenteroides* ssp *cremoris* LAB5 (Kolapo et al., 2019) were maintained on relevant agar slopes in the refrigerator.

### Preparation of Soybean Daddawa by Natural and Starter Culture Fermentation

Preparation of soybean daddawa by Natural fermentation was done following the traditional method of Popoola and Akueshi (2005). In another approach, seven batches of fermented product were produced in a controlled setting replacing the rudimentary equipment used in the traditional method with glassware as described earlier by Kolapo et al. (2019). Starter cultures selected on the basis of their previously described technological characteristics were introduced into the fermentation medium at the onset of fermentation. They were used as a monoculture, double cultures and multiple cultures resulting in seven treatments of controlled fermentation. Triplicate samples were withdrawn for analyses at 0, 24, 40 and 65 hours of fermentation for enzymatic, chemical and functional analyses.

### Assay of Enzyme Activities of the Fermenting Soybean Daddawa

Enzyme extract of the fermenting soybean seeds was prepared by grinding 5 g of sample in 50 ml of 0.1 M Phosphate buffer, pH 6.5 as the extracting buffer. The suspension was washed with petroleum ether (Aldrich 26,173-4) to extract the oil and centrifuged at 4000 rpm for 5 min (Yong and Wood, 1977). The supernatant constituting the crude enzyme extract was stored at $-40^0$C and subsequently used for protease and alpha amylase activities determinations. Protease activity was determined as described by Dakwa et al. (2005). One unit of protease activity was defined as the amount which produced 1.0 µmol of tyrosine in 1.0 ml of the trichloroacetic acid-soluble peptides under assay conditions. Alpha amylase activity was determined by the assay method of Bernfeld (1955).

### Determination of Free Amino Acids and Total Soluble Sugars of the Fermenting Soybean Daddawa

Eighty per cent ethanol (v/v) was used to extract the free amino acid and total soluble sugar content of the fermenting soybean-daddawa using the method of Odibo et al. (1990). The total free amino acid content was determined by the ninhydrin colorimetric method of Rosen (1957) as described by Dakwa et al. (2005) and the free amino acids were determined by correlating absorbance at 420 nm with a standard of glycine. The total soluble sugar was determined by the anthrone reagent method of Morris (1948) as described by Omafuvbe et al. (2002). The total soluble sugar was determined by correlating absorbance at 625 nm with a standard of glucose.

### Determination of pH and Titratable Acidity of the Fermenting Soybean Daddawa

A modification of the method described by Sanni (1988) was employed in measuring the pH of the fermenting samples. Five grammes of the sample were weighed into the mortar and grounded. Slurry was made by adding 45ml of distilled water in order to obtain a one tenth dilution. The pH value of the slurry was obtained using a pH meter whose electrode had been standardized to both acidic and alkaline pH.

The method described by Ikenebomeh et al. (1986) was employed to estimate the titratable acidity of the fermenting samples. Five grammes of the sample were macerated in 45ml decarbonated distilled water. The slurry was filtered through a fine muslin cloth and 10ml aliquot of the filterate was titrated with 0.1M NaOH with phenolphthaline as the end point indicator. Ten millilitres of decarbonated water was also titrated and the water titre subtracted from the sample titre. One millilitre of 0.1 N NaOH was taken as equivalent to 9.008 x $10^{-3}$g lactic acid. Titratable acidity was calculated as mg lactic acid per g of sample (mg/g).

**Determination of Water Absorption and Fat Absorption Capacities of the Fermenting Soybean Daddawa**

The method of Sosulki et al. (1976) was used for both water and fat absorption capacities measurement. Five grammes of sample were grinded to make slurry in 15 ml distilled water. The slurry was centrifuged at 4,000 x g for 20 min. The free water was decanted and the amount of absorbed water was determined by difference. The same methodology was used for fat absorption, except that the soybean daddawa and oil in the centrifuge tube was stirred for 30s at 5 minutes interval and centrifugation was done after 30 minutes.

**Statistical Analysis**

Data obtained were expressed as means. Analysis of variance was carried out on the data obtained to determine significance of differences. A two-tailed P value of less than 0.05 was considered to be statistically significant. Values that were significantly different were separated using Duncan Multiple Range test using SPSS for windows Verson 11.0 statistical package.

## RESULTS AND DISCUSSION

**Protease and α-amylase Activities in the Fermenting Soybean Daddawa.**

The fermentations inoculated with mono and mixed cultures of the starter cultures exhibited different degree of protease activity (Figures 1 a, b and c). A two way analysis of variance depicted that both fermentation time and the type of culture employed in the fermentation process had significant effect ($p<0.05$) on the protease activity in the fermenting soybean daddawa. The protease activity continued to increase in the monoculture fermentation from the start of the fermentation until the 65$^{th}$ hour of the fermentation. However, in the mixed culture fermentations including the system that employed the three organisms, optimum protease activities were reached at the 40$^{th}$ hour of fermentation which incidentally coincides with the time that the significant noticeable change was being observed in the natural fermentation set-up. Among the monoculture fermentations, *Staphylococcus xylosus* exhibited the highest level of proteolytic enzyme activity while *Leuconostoc mesenteroides ssp cremoris* had the least activity.

Figures 2 a, b and c show that α-amylase activities in the fermentations inoculated with mono and mixed starter cultures differed. A two way analysis of variance depicted that both fermentation time and the type of culture employed in the fermentation process had significant effect ($p<0.05$) on the α-amylase activities in the fermenting soybean daddawa. In both the mono and mixed culture fermentation, α-amylase enzyme activities generally increased with increasing fermentation time. The α-amylase enzyme activities continued to increase in the monoculture fermentation from the start of the fermentation until the 65$^{th}$ hour of the fermentation. However, in the mixed culture fermentations which employed *Bacillus subtilis/Staphylococcus xylosus* and *Staphylococcus xylosus/Leuconostoc mesenteroides ssp cremoris* the optimum amylase activities were reached at the 40$^{th}$ and 65$^{th}$ hour of -fermentation respectively. The combination of *Bacillus subtilis* and *Leuconostoc mesenteroides ssp cremoris* produced a progressive decrease in amylase activity up to the 40$^{th}$ hour of fermentation after which it increased to the peak in 65 h of fermentation. However, in the fermentation which employed the three bacteria, optimum

amylase activity was achieved in 40 h while that of natural fermentation was 65 h.

**Free Amino Acids and Total Soluble Sugars Changes in the Fermenting Soybean Daddawa.**

Figures 3 a, b and c show the changes in the free amino acid (FAA) contents of the fermenting soybean daddawa inoculated with mono and mixed cultures of the starter cultures. A two way analysis of variance depicted that both fermentation time and the types of culture employed in the fermentation process had significant effect ($p<0.05$) on the free amino acid contents of the fermenting soybean daddawa. In both the mono and mixed culture fermentation, free amino acid contents generally increased with increasing fermentation time but the pattern of FAA changes differed based on the nature of starter cultures employed.

Figures 4 a, b and c show the changes in the total soluble sugars (TSS) contents of the fermenting soybean daddawa inoculated with mono and mixed cultures of the starter cultures. A two way ANOVA test revealed that both fermentation time and the culture types used in the fermentation process had significant effect ($p<0.05$) on the TSS contents of the fermenting soybean daddawa. TSS increased with increasing fermentation time in both the mono and mixed culture fermentation.

**pH and Titratable Acidity Changes in the Fermenting Soybean Daddawa.**

Figures 5 a, b and c show the changes in the pH of the fermenting soybean daddawa inoculated with mono and mixed cultures of the starter cultures as well as naturally fermented daddawa. In both the mono and mixed culture fermentation, pH generally increased with increasing fermentation time. However, the pattern of pH changes differed with the type of starter cultures employed. In the control fermentation set-up which employed mono and two starter cultures, alkaline pH in the neighbourhood of 8.0 rapidly developed within 24 h of fermentation. However, in the control fermentation which used three starter bacteria, similar alkaline pH developed only at the 40$^{th}$ hour of fermentation. At the 65 h of fermentation, natural fermentation was not able to produce high pH (~8.5) which was characteristic of all the fermented soybean daddawa obtained through optimized procedures (Figures 4 a, b and c).

The titratable acidity changes (TA) in both controlled and natural fermentation of soybean daddawa is shown in Figures 6 a, b and c. In all the fermenting soybean daddawa, there was significant increase ($p<0.05$) in the TA developed between the start and the end of the fermentation. The pattern of TA changes in the naturally fermented soybean daddawa was perfectly replicated in the control fermentation mediated by three-member starter cultures, only that the acidity developed in the latter was significantly higher ($p<0.05$).

**Water and Fat Absorption Capacities Changes in the Fermenting Soybean Daddawa.**

Table 1 shows the water and fat absorption capacities of soybean daddawa produced by mono and mixed starter cultures as well as natural fermentation. Both fermenting organism type and the fermentation time had significant effect ($p<0.05$) on the water absorption capacities (WAC) of the fermenting soybean daddawa. On the contrary, the fat absorption capacity of the fermenting soybean daddawa was not significantly ($p>0.05$) affected both by the fermenting organism type and the fermentation time.

In all fermentation set-ups the WAC of the fermenting mash increased with fermentation time. However, the extent of increase depended on the nature of the culture use in the fermentation process. The monoculture fermentation resulted in the highest increase with the exception of *Staphylococcus xylosus* fermented mash which resulted in the least increase.

**DISCUSSION**

Achi (2005) stated that the use of mixture of microorganisms with complimentary physiological and metabolic properties seems to be the best approach for obtaining a product with the nutritional and sensory properties desired. Results from the present study showed that the observed protease activity in multiple

culture fermentation set-ups were significantly higher than the observed values in the monoculture fermentation. This appears to suggest that a form of synergism is associated with protease activity in multiple culture fermentation. Similar observation was made by Omafuvbe et al. (2002) wherein the presence of *B. subtilis* in the mixed starter fermentation resulted in increased proteolytic activity. In addition to the afore-stated positive development, optimum protease activities were reached within 40 h fermentation in multiple culture fermentation while it took 65 h in mono culture fermentation. From economic point of view, the optimization process which employs multiple cultures would be most profitable.

In a trend that is contrary to the protease activity, α-amylase activity of the fermenting daddawa appeared to be lowered with increasing number of microorganisms used as components of mixed starter cultures. The optimum amylase activities in virtually all mixed starter culture fermentation were reached at 65 h of fermentation. However, the observed lowered α-amylase activity associated with multiple culture fermentation may not obliterate the earlier gains of multiple culture fermentation on the understanding that soybean fermentation is essentially an alkaline fermentation.

Omafuvbe et al. (2002) reported that in both natural and controlled fermentation of soybean daddawa, Free Amino Acid (FAA) content continued to increase between 24$^{th}$ and 72th hour of fermentation; an event that was preceded by intense protease activity. The result obtained in the present study is in agreement with this observation as significant accumulation of FAA in all the fermentation set-ups took place between 24$^{th}$ and 65$^{th}$ hour of fermentation. The pattern of change in the FAA contents of the fermenting soybean daddawa in the present study is in agreement with previous reports of Aderibigbe and Odunfa (1990) and Omafuvbe et al. (2002) whereby increased protease activity resulted in increased FAA contents. Among the monoculture fermentation, *Staphylococcus xylosus* fermented soybean daddawa which had earlier exhibited highest protease activity resulted in the highest mean FAA contents. Similar results were obtained in the multiple culture fermentation which made use of three starter organisms and those of natural fermentation. However, the above observed trend was not exhibited in the *Bacillus subtilis*-fermented daddawa and other mixed culture fermentations. This somewhat unusual trend may be consequent upon the associated metabolic changes. Amino acids are doubtlessly the end product of protease activity. The fate of such amino acids includes any of the following. Allagheny et al. (1996) stated that the production of ammonia in a fermenting soybean daddawa is a consequence of the utilization of amino acids by the fermenting bacteria as sources of carbon and energy. In another development, Strecker degradation of amino acids results into the formation of α-amino ketones which in turn can condense with α-dicarbonyl compounds to form pyrazzine (MacLeod and Ames, 1988), which is an important flavour compounds. Therefore, in a fermentation set-up where increased protease activity did not positively correlate with FAA, it is possible that the rate of metabolism/utilization of amino acids exceeded its production rate through proteolysis.

The pattern of change in the total soluble sugar (TSS) contents of the fermenting soybean daddawa in the present study is in agreement with previous reports of Omafuvbe et al. (2002) whereby increased amylase activity with fermentation time resulted in increased TSS contents. In all the fermentation set-ups, *Bacillus subtilis*-fermented soybean daddawa had the highest amylase activity but it surprisingly had the least mean TSS. This paradox might be a reflection of greater utilization/metabolism of sugars released by amylase activity. Studies have shown that a number of amino acids readily react with monosaccharides to form alkylpyrazines (Rizzi, 1987; Whitfield, 1992). Leahy and Reineccius (1989) suggested that the formation of pyrazines is favoured by free ammonia. In a related development, Owens et al. (1997)

submitted that the combination of alkaline pH (~8.0), high ammonia concentration and free amino acids in the fermentations allowed the formation of pyrazines at the $35^0$C incubation temperature. Interestingly, it was only in *Bacillus subtilis*-fermented soybean daddawa that there was rapid pH increase to above 8.0 within 24 h of fermentation. It is therefore possible that the utilization of both amino acids and monosaccharides for the formation of pyrazines most likely accounted for the lower level of TSS in *Bacillus subtilis*-fermented soybean daddawa despite its highest amylase activity.

In legume alkaline fermentation the major metabolic activity of the bacteria is proteolysis of the legume protein and utilization of the released amino acids (Allagheny et al., 1996). Consequentially, ammonia is formed (Ohta, 1986; Steinkraus, 1991; Sarkar et al., 1993) and the pH value rises. The rise in pH with fermentation time as observed in the present study compares favourably with previous reports on legume fermentation (Ohta, 1986; Steinkraus, 1991; Sarkar et al., 1993; Omafuvbe et al., 2002). The kinetics of pH increase observed in *Bacillus subtilis*-fermented soybean daddawa is quite different from other fermentation set-ups. In the daddawa fermented by *B. subtilis* pH increased rapidly to above 8.0 in the first 24 h of fermentation while similar pH value was attained in other controlled fermentation set-ups but at a much latter hours of fermentation. Considering the fact that the protease activity in the *Bacillus subtilis*-fermented soybean daddawa was not particularly higher than other set-ups within the first twenty-four hours of fermentation, it then seems likely that within 24 h of fermentation the rate of metabolism of amino acids liberated by proteolysis was relatively higher in daddawa fermented with *B. subtilis*. However, in other set-ups including natural fermentation, amino acid utilization which normally results in pH increases was a latter development.

The simultaneous increase in pH and titratable acidity observed in the present study as fermentation progressed has been reported in the fermentation of similar foods (Wagenknecht et al., 1961; Ikenebomeh, 1989; Omafuvbe et al., 2000). In these studies it was suggested that liberated ammonia or other basic end products of protein decomposition were the cause of pH increase. Frazier and Westhoff (1978) opined that the carbohydrate component of a foodstuff is usually hydrolyzed to simple sugars which may be fermented to produce organic acids. Therefore, increase in titratable acidity observed in the present study might be consequent on sugar metabolism which resulted into the formation of organic acids which gave rise to increased acidity. On the other hand, increased pH was consequent on metabolism of amino acids earlier liberated through proteolysis.

The values for water absorption capacity (WAC) obtained in the presented study are higher than the value of 210 % reported by Obatolu et al. (1998) for naturally fermented soybean daddawa. Giami and Bekeham (1992) stated that fermentation does not affect WAC in legumes. However, the result of the present study revealed that fermentation (whether controlled or spontaneous) actually resulted in increased WAC. Water absorption capacity is an indication of a food product to associate with water in conditions where water is limiting. In a related development, the values for fat absorption capacity (FAC) were favourably comparable to the values of 0.84 - 1.5 ml/g reported by Lin et al. (1974) for sunflower meal products. Fat absorption capacity (FAC) could be attributed to the physical entrapment of oils (in the seeds) which is related to number of non-polar side chains on the proteins that bind hydrocarbon chains of the fatty acids. This functional attribute of foods could be important as the ability of the proteins to bind fats is important since fat acts as flavour retainer and increases mouth feel of foods. Results from this study have shown that controlled fermentation of soybean daddawa can still produce daddawa of acceptable FAC values.

Meanwhile, our recent report (Kolapo et al. 2019) indicated that multiple starter cultures fermentation of soybean daddawa led to the

production of daddawa that was next rated to naturally fermented samples in term of overall acceptability and that wide arrays of enzyme was generated by the ‘‘cocktail’’ of fermenting organisms such that was not furnished by the individual starter organism.

## CONCLUSION

The involvement of *Staphylococcus* spp. and LAB in alkaline fermentation has been described to be unusual. Results from the present study have shown that there is a need for paradigm shift from this long-held position if the natural aroma of spontaneously fermented daddawa is to be replicated in a controlled setting. In the present study, multiple culture fermentation which comprised *Bacillus subtilis, Staphylococcus xylosus* and *Leuconostoc mesenteroides* ssp *cremoris* resulted in an increased proteolytic activity in the fermenting daddawa mash. The proteolytic activity observed in the multiple culture fermentation was higher than that of monoculture fermentation but comparable to that of natural fermentation. This holds a great promise for subsequent industrialization of soybean daddawa production as natural fermentation of soybean daddawa is nearly replicated in a

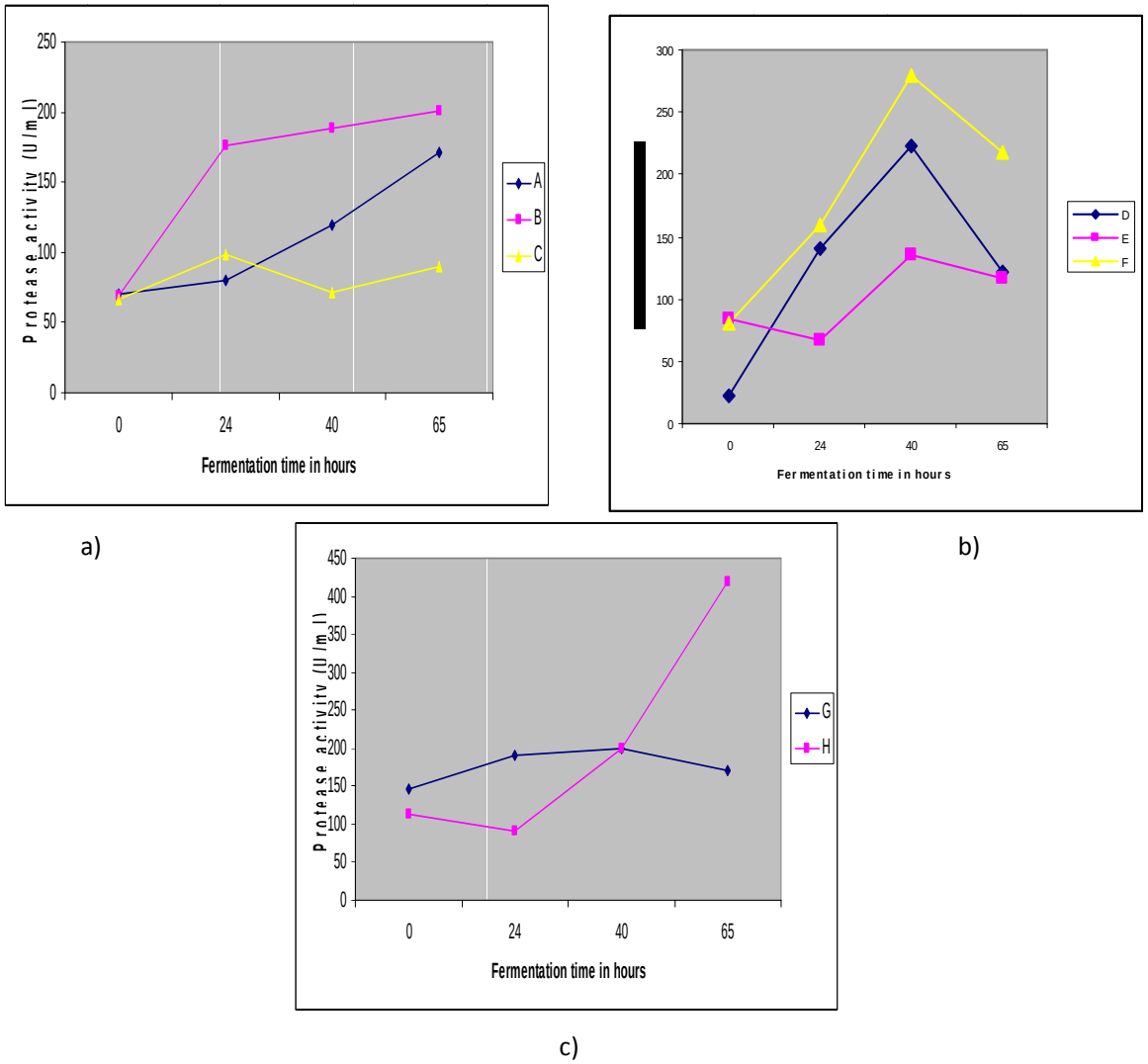



**Figure 1** Protease activities in fermenting soybean daddawas

**KEY FOR FIGURES 1 -6**

A. Fermentation with *Bacillus subtilis*
B. Fermentation with *Staphylococcus xylosus*
C. Fermentation with *Leuconostoc mesenteroides ssp cremoris*
D. Fermentation with *Bacillus subtilis* and *Staphylococcus xylosus*
E. Fermentation with *Bacillus subtilis* and *Leuconostoc mesenteroides ssp cremoris*
F. Fermentation with *Staphylococcus xylosus* and *Leuconostoc mesenteroides ssp cremoris*
G. Fermentation with *Bacillus subtilis* +*Staphylococcus xylosus* + *Leuconostoc mesenteroides ssp cremoris*
H. H-Natural fermentation

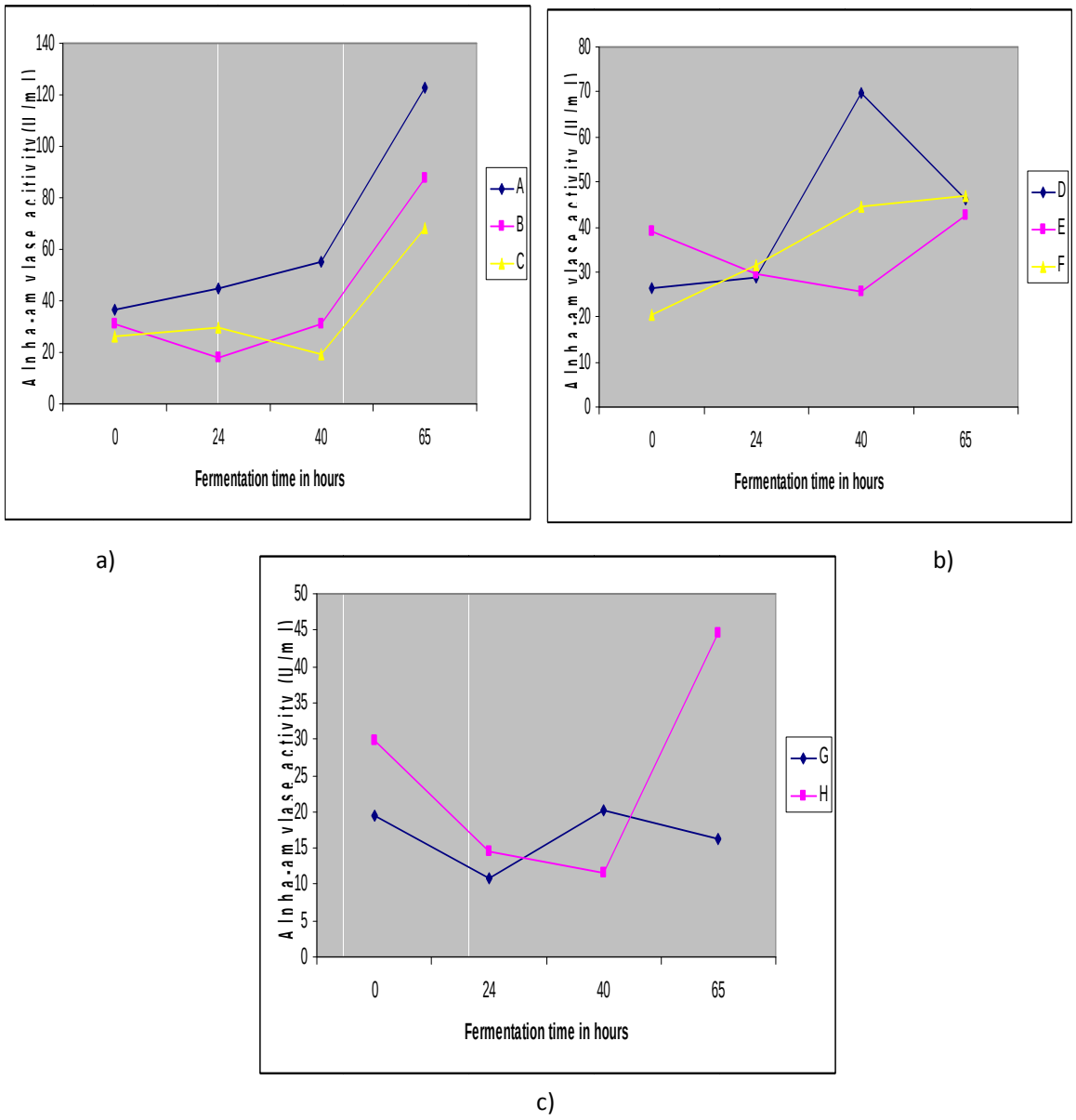



**Figure 2**. Alpha amylase activities in fermenting soybean daddawa

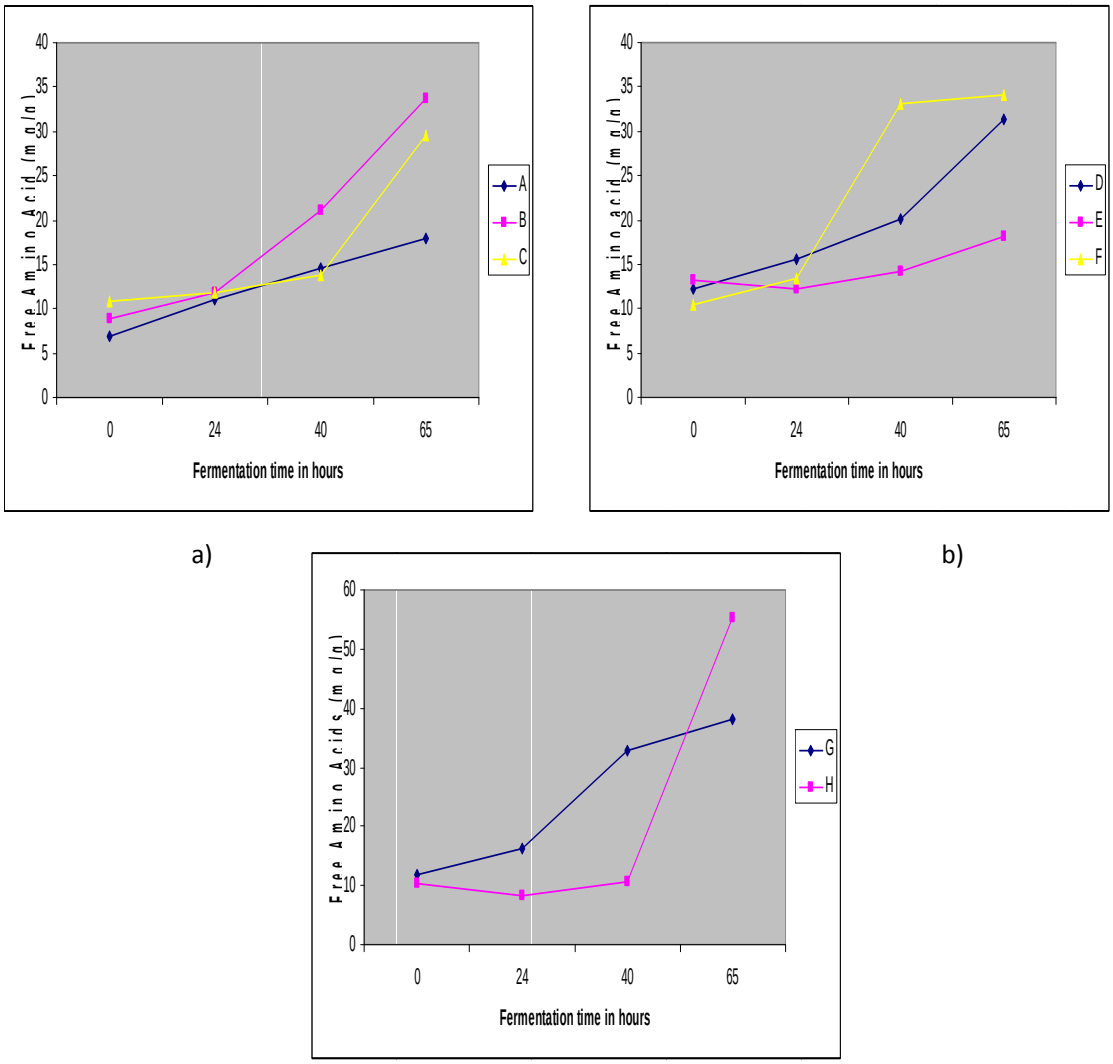
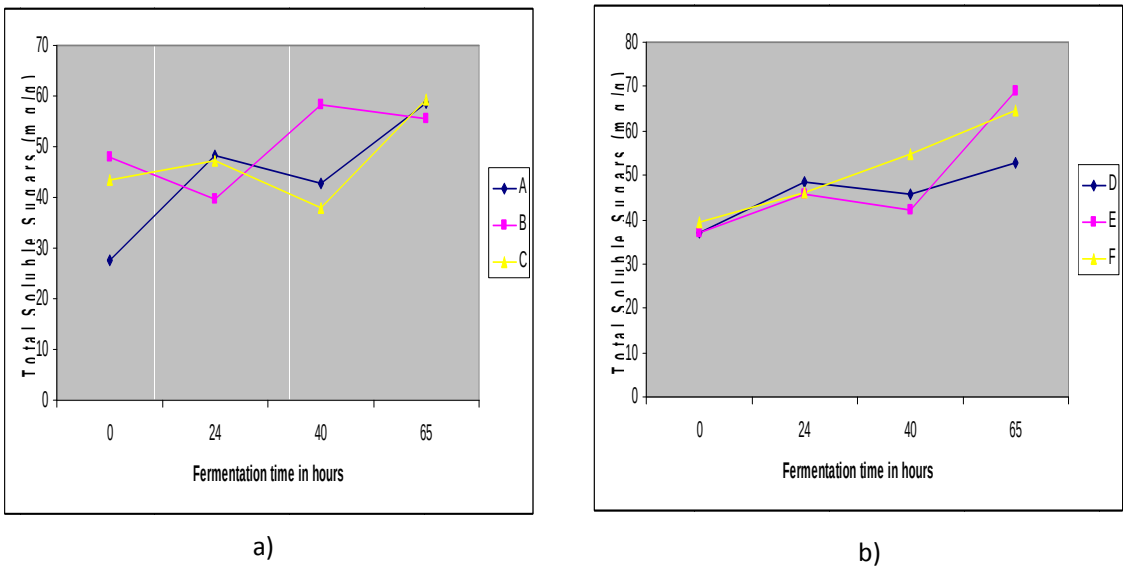
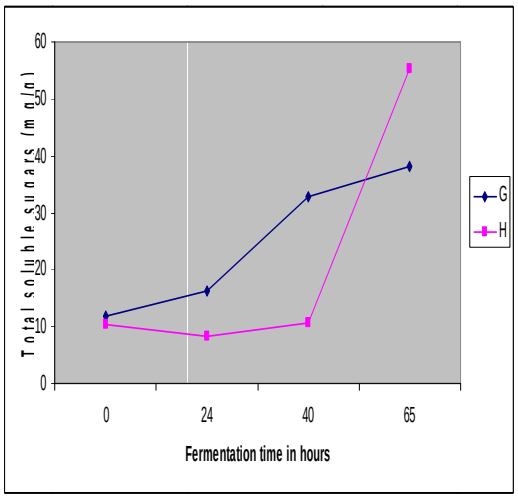


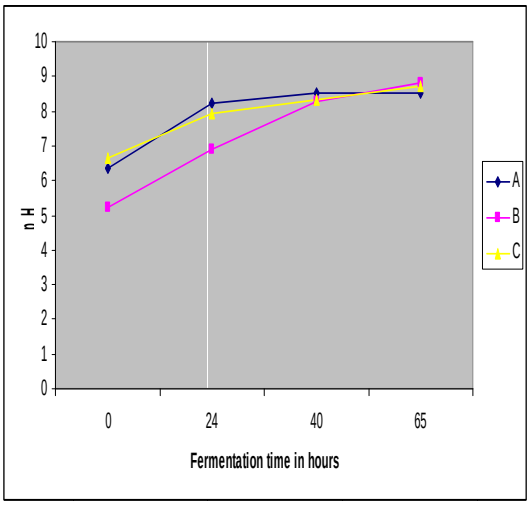

**Figure 3.** Total free amino acid in fermenting soybean daddawa



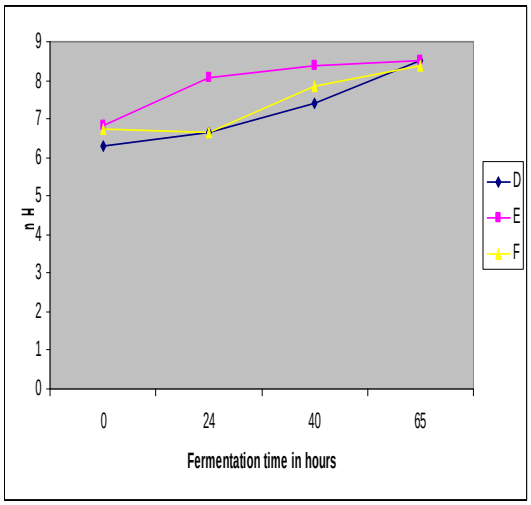



c)

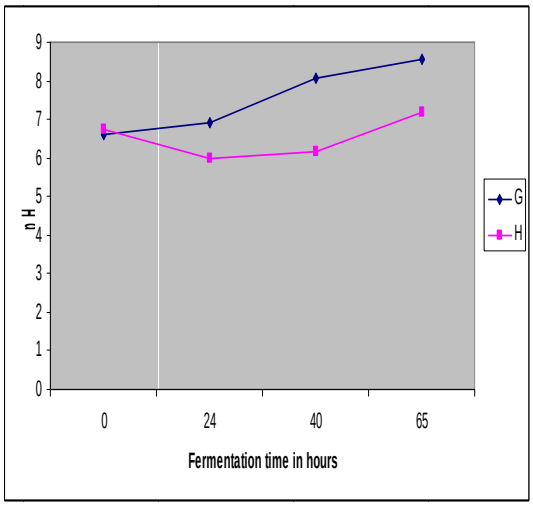
**Figure 4.** Total soluble sugars in fermenting soybean




a)




b)




c)

**Figure 5**. Changes in pH of fermenting soybean daddawa

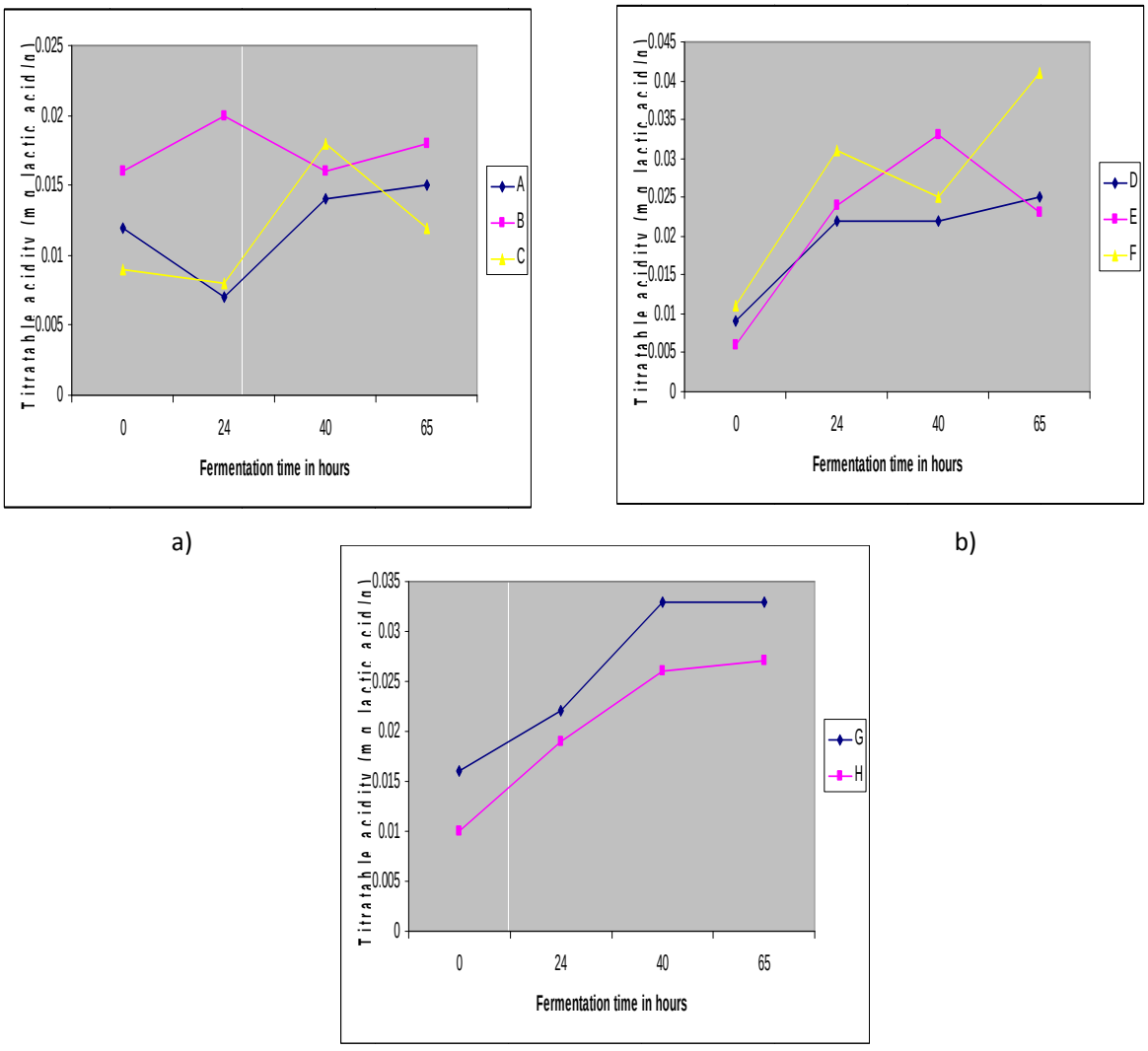



**Figure 6.** Changes in titratable acidity of fermenting soybean daddawa

**Table 1** Water and Fat Absorption Capacities of Fermenting Soybean Daddawa

| | Water Absorption Capacity (%) | | Fat Absorption Capacity (ml/g) | |
|---|---|---|---|---|
| | Fermentation Time(h) | | Fermentation Time(h) | |
| Sample | 0 | 65 | 0 | 65 |
| A | 260 $^{b}$ | 390$^{a}$ | 2.0 $^{a}$ | 1.5 $^{a}$ |
| B | 290 $^{a}$ | 300 $^{a}$ | 1.5 $^{a}$ | 1.5 $^{a}$ |
| C | 240 $^{b}$ | 330 $^{a}$ | 1.0 $^{a}$ | 1.5 $^{a}$ |
| D | 300 $^{a}$ | 330 $^{a}$ | 1.5 $^{a}$ | 1.0 $^{a}$ |
| E | 220 $^{b}$ | 320 $^{a}$ | 1.5 $^{a}$ | 1.5 $^{a}$ |
| F | 250 $^{b}$ | 300 $^{a}$ | 1.5 $^{a}$ | 1.5 $^{a}$ |
| G | 210 $^{b}$ | 250 $^{a}$ | 2.0 $^{a}$ | 1.5 $^{a}$ |
| H | 230 $^{b}$ | 290 $^{a}$ | 2.0 $^{a}$ | 1.5 $^{a}$ |

Values are means of triplicate determinations. Along row, values with different superscripts are significantly different ($P<0.05$)

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