MICROORGANISMS WITH IMPROVED SACCHAROGENIC AND PROTEOLYTIC PROPERTIES FOR ROOT CROP-BASED SOY SAUCE

Julie C. Diamante

Assistant Professor, Philippine Root Crop Research and Training Center (PRCRTC), ViSCA, Baybay, Leyte, Philippines.

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ABSTRACT

Mold isolates coded as RT, JJ and LL and identified as *Aspergillus flavus* Link. var. *columnaris* var. nov., *A. niger* van Tieghem and *A. phoenicis* (Cda.) Thom., respectively; when used in soy sauce production; yielded high amounts of sugar and protein. The appearance, aroma, color, flavor and general acceptability of root crop-based soy sauce produced by *A. flavus* var. *columnaris* were comparable to those produced by *A. sojae* and *A. oryzae*. However, only the aroma of the soy sauce produced by *A. phoenicis* using sweet potato flour was comparable to the control and that produced by *A. flavus* var. *columnaris*. When cassava flour was used; appearance, aroma and color were comparable to the control soy sauce and that produced by *A. flavus* var. *columnaris*. *A. niger* produced the least acceptable soy sauce in terms of all sensory qualities considered when sweet potato flour was used. With cassava flour, the aroma and flavor of the product were comparable to the control and that produced by *A. flavus* var. *columnaris*. Aflatoxin was not detected in any soy sauce produced by the test microorganisms.

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KEY WORDS: Root crop-based soy sauce. *Aspergillus* spp. Saccharogenic and proteolytic activities. Chemical composition. Sensory qualities.

INTRODUCTION

Soy sauce is the most widely consumed fermented product from the Orient. It is commercially prepared from a combination of soybeans and flour with salt through the action of microorganisms. Tested strains of *Aspergillus oryzae* and *A. sojae* such as NRRL-1988 and 1989 are used as starter cultures (Wang and Hesseltine, 1982). To accelerate fermentation and improve the flavor of the final product, pure cultures of yeasts and bacteria such as *Saccharomyces rouxii*, *Torulopsis* and *Pediococcus soyae* are inoculated into the mash.

Several microorganisms can accelerate fermentation and contribute flavors in soy sauce production. A special type of microorganism known as *Aspergillus oryzae* was used by Galvez (1980) and this microorganism when used with either wheat or root crop flour produced less acceptable soy sauce than the commercial ones. Another microorganism, *Aspergillus sojae* obtained from Japan was also tried by Data et al.(1983). The flavor of the product was comparable to commercial brands but its color, aroma and consistency were not. Soriano (1978 as cited by Joson, 1978) used the yeast *Hansenula anomala* as first inoculant to cooked soybeans then added with *A. oryzae* and *Lactobacillus delbruekii* after 24 hours. Good quality soy sauce was produced after 6 weeks of fermentation. Mendoza (1961) suggested the use of sliced radish and shredded violet-colored cabbage which naturally contain the desired microorganisms for soy sauce production instead of a pure bacterial culture. Moreover, a mixed microflora of lactic acid bacteria such as *Streptococcus* and *Lactobacillus* for soy sauce production was developed to make the fermenting mash acidic. *Bacillus* sp. was also reported to grow in the mash (Frazier, 1967).

This study aimed to isolate microorganisms from fermenting or decaying food items in different areas of Leyte, to test the saccharifying and proteolytic power of the isolates in soy sauce production using root crop flour, to evaluate the chemical and sensory qualities of soy sauce produced from high protein-producing isolates, and to compare the qualities of soy sauce produced using the promising isolates with those produced using *A. oryzae* and *A. sojae*.

MATERIALS AND METHODS

Isolation and Purification of Microorganisms

Microorganisms were isolated from naturally fermented food items such as decayed fruits, vegetables, cereals and root crops obtained from different areas of Leyte. The isolates were grown in different media (potato dextrose agar (PDA) for molds, malt yeast agar (MYA) for yeasts and glucose yeast peptone (GYP) for bacteria), purified and classified into their respective groups.

Screening of Microorganisms for Saccharogenic and Proteolytic Power

Pure cultures were individually screened for their saccharogenic power using the Fehling Leaman Schoorls method and proteolytic power using the protein hydrolysis index method.

Saccharogenic power. Yeasts, molds and bacteria were grown in malt yeast (MY), potato dextrose (PD) and glucose yeast (GY) broth, respectively for 1 day at 30°C under agitated condition. The solution with microbial cells was centrifuged for 5 min at 3000 rpm. One mL of the liquid portion (enzyme solution) was added to 10 mL of 2% starch solution and 2 mL of acetate buffer solution (pH 5). The resulting reaction mixture was incubated at 40°C for 70 min after which 7 mL of 0.1 N NaOH solution was added to stop enzymatic reaction.

The sugar content was determined through titration of the $CuSO_4$–NaK tartrate sample solution with $Na_2S_2O_3$ until the iodine-starch reaction color disappeared or the solution turned milkish white.

The amount of sugar was calculated as follows:

$$(V_1 - V_2)\,F = X\ (\text{mL})$$

Where

V_1 = volume of titrant used for blank titration

V_2 = volume of titrant used for samples titration

F = factor of $Na_2S_2O_3$

$$\text{Sugar (\%)} = Y \times \frac{1}{1000} \times \frac{100}{\text{sample (mL)}}$$

Where Y = sugar (mg) from Fehling Leaman Schoorls table by x (mL)

Proteolytic Power. A 2 mm thick (approximately 12 mL) melted mixture of standard method caseinate agar (SMCA) was poured into petri dishes. When the medium solidified, the microorganisms were spot inoculated and incubated at 30°C for 48 hr. The organisms that formed white or off-white precipitates around the colony on SMCA were considered proteolytic. Protein hydrolysis index (phi) was measured as the ratio of halo to colony diameter and indicates the proteolytic activity of the microorganisms.

Evaluation of Soy Sauce Produced by Promising Isolates

Sugar and protein contents of soy sauce were determined using the Fehling Leaman Schoorls method and microkjeldahl method, respectively every 15 days of fermentation up to 3 months. Aflatoxin production was determined after 3 months of fermentation. Sensory evaluation in terms of appearance, aroma, color, flavor and general acceptability was done on the different soy sauce produced using the selected isolates and the control microorganisms (*A. sojae* and *A. oryzae*). Soy sauce was served alone and was tasted pure.

Identification of Isolates

The promising isolates were grown in Czapek dox agar (CZD) and malt extract agar (MEA) and then identified according to their morphological characteristics. The growth of the isolates was characterized macroscopically and microscopically from 1 to 14 days of incubation at 28° to 30°C based on characteristics de scribed by Raper and Fennell (1965) and Rayner (1970). Each solate was differentiated based on color, colony growth, size and form of conidial head, conidiophore, vesicle, sterigma and conidium.

RESULTS AND DISCUSSION

Isolation and Purification of Microorganisms

A total of 195 microorganisms of different types, species and/or strains was isolated. Bacteria were the most prevalent microorganisms in naturally fermented food items. They constituted 44% of the total isolates followed by yeasts (37%) and molds (29%).

Screening of Microorganisms for Saccharogenic Power

Generally, molds have higher saccharogenic power than some yeasts and bacteria (Table 1). Twenty three percent of the molds had saccharogenic power within the range of 30.1 to 40.0% which was the highest whereas only 7% of the yeasts and none of the bacteria attained this range. On the other hand, 48 and 36% of molds and yeasts, respectively had saccharogenic power between 20.1 and 30.0%. All bacteria had saccharogenic power of 20% and below.

Table 1. Saccharogenic power of microbial isolates.

Saccharogenic Power (%)	Percent Isolates		
	Molds	Yeasts	Bacteria
0.0	0	4	47
0.1 - 10.0	11	38	46
10.1 - 20.0	18	15	7
20.1 - 30.0	48	36	0
30.1 - 40.0	23	7	0

Evaluation of Promising Isolates for Saccharogenic Power

Table 2 shows the saccharogenic power of two isolates each of molds, yeasts and bacteria and their sources compared with the control, *A. sojae* and *A. oryzae*. *A. flavus* var. *columnaris*, *A. phoenicis* and yeasts (S1 and T1) exhibited higher saccharogenic power than the control and the bacterial isolates (P2 and BB).

It is worth noting that S1 was isolated from radish and this finding supported the recommendation of Mendoza (1961) to add sliced radish in soy sauce production. This food material naturally contained the microorganisms responsible for the development of the characteristic flavor of soy sauce. This is applicable especially in small-scale production where the preparation and use of pure culture would be impractical.

Screening of Microorganisms for Proteolytic Power

Some bacterial species have high protein degradation capacity as indicated by their higher protein hydrolysis indices (phi) compared to those of yeasts and molds (Table 3). Other bacterial species, however, have lower phi values than some molds and yeasts. Moreover, an undefined relationship between saccharogenic power and phi values was observed. This implies that microorganisms with high capacity to degrade starch into simple sugars may or may not necessarily be responsible for protein degradation particularly into soluble forms.

Evaluation of Promising Isolates for Proteolytic Power

Sweet pepper contained a mold with both high proteolytic (Table 4) and saccharogenic power which was identified as *A. phoenicis*. All

Table 2. Isolates with high saccharogenic power and their sources.

Isolates		Saccharogenic power[1] (%)	Source
Molds[2]	(RT) *A. flavus* Link var. *columnaris* var. nov.	39.58 a	Red tuba
	(JJ) *A. phoenicis* (Cda.) Thom.	37.36 a	Sweet pepper
Yeasts	S1	34.60 a	Radish
	T1	29.54 ab	Baguio beans
Bacteria	P2	16.50 c	Carrot
	BB	11.79 c	Irish potato
Control	*A. sojae*[3]	21.58 bc	
	A. oryzae[4]	21.70 bc	

[1] Means followed by a common letter are not significantly different at 5% level, DMRT.
[2] Microorganisms identified at PRCRTC laboratory.
[3] Obtained from Japan as pure culture.
[4] Obtained from UPLB as pure culture.

Table 3. Protein hydrolysis index (phi) values of the isolates.

Protein Hydrolysis Index	Molds	Yeasts	Bacteria
0.00	42	83	35
0.01 - 0.99	0	0	0
1.00 - 1.99	58	11	32
2.00 - 2.99	0	2	7
3.00 - 3.99	0	2	15
4.00 - 4.99	0	2	10
5.00 - 5.99	0	0	1
Total (%)	100	100	100

other isolates especially yeasts and bacteria had high proteolytic power but not necessarily high saccharogenic power. Most of the common molds possess various hydrolytic enzymes such as amylases, proteinases, lipases and pectinases (Frazier and Westhoff, 1978). Unlike some yeasts and bacteria, one mold species could solely be responsible for carbohydrate and

Table 4. Isolates with high proteolytic power and their sources.

Isolate		Proteolytic Power (phi)[1]	Source
Molds[2]	(JJ) *A. phoenicis* (Cda.) Thom.	1.76 c	Sweet pepper
	(LL) *A. niger* van Tieghem	1.69 c	Cucumber
Yeasts	A	4.20 a	Sweet potato
	Q2	3.00 b	Onion
Bacteria	X	5.00 a	Eggplant
	PP	4.46 a	Chayote
Control	*A. sojae*[3]	2.00 c	
	A. oryzae[4]	1.93 c	

[1] Means followed by a common letter are not significantly different at 5% level, DMRT.
[2] Microorganisms identified at PRCRTC laboratory.
[3] Obtained from Japan as pure culture.
[4] Obtained from UPLB as pure culture.

protein degradation, a combined process necessary in soy sauce production. This observation must be supported by data on the chemical and sensory characteristics of its product to determine the acceptability of the said mold in soy sauce production.

Chemical Analysis of Root Crop-Based Soy Sauce

When grown singly for soy sauce production, mold isolates including the control produced more sugar and protein compared to yeasts and bacteria after fermentation (Table 5). *A. niger* and *A. phoenicis* produced relatively higher amounts of sugar than *A. flavus, A. sojae* and *A. oryzae*. All yeasts and bacteria produced markedly low amounts of sugar and protein. This implies that microorganisms with high proteolytic power like yeasts and bacteria may not necessarily produce high amounts of protein in soy sauce.

The amounts of protein and sugar in soy sauce produced by the microorganisms from 0 to 90 days of fermentation using sweet potato are shown in Figure 1. The protein content of soy sauce produced by *A. sojae, A. oryzae* and *A. flavus* was comparable to each other from 45 to 90 days of storage although the highest amount was produced by *A. flavus* after 90 days of storage. Using *A. oryzae* however, the highest amount of protein was produced at 60 days; the amount progressively decreased until 90 days of fermentation. *A. niger* and *A. phoenicis* produced relatively low amount of protein during the entire fermentation period. Using *A.*

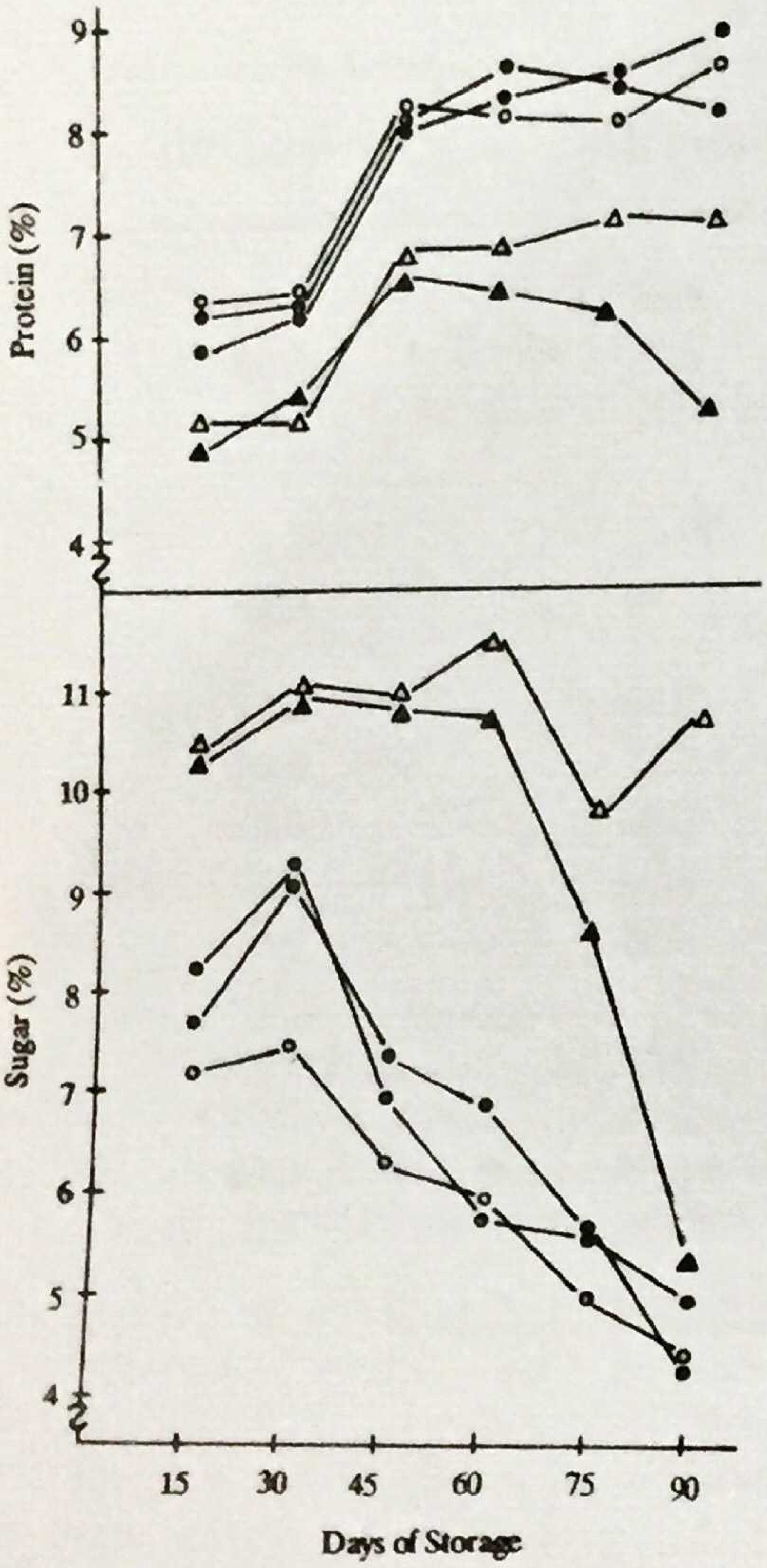
Table 5. Sugar and protein contents of soy sauce produced by different microorganisms after one month of fermentation.

Microorganism		Sugar (%)	Protein (%)
Molds	*A. sojae*[1]	8.28	5.38
	A. oryzae[1]	8.36	5.36
	(RT) *A. flavus* Link var. *columnaris* var. nov.[2]	8.81	5.01
	(LL) *A. niger* van Tieghem[2]	10.42	4.94
	(JJ) *A. phoenicis* (Cda.) Thom[2]	12.14	4.79
Yeasts	*Saccharomyces rouxii*[1]	0.34	0.01
	A	0.56	0.01
	T1	0.90	0.01
	S1	0.68	0.01
	Q2	0.84	0.01
Bacteria	BB	0.80	0.01
	P2	0.58	0.01
	X	0.57	0.01
	PP1	0.66	0.01

[1] Control
[2] Microorganisms identified at PRCRTC laboratory.

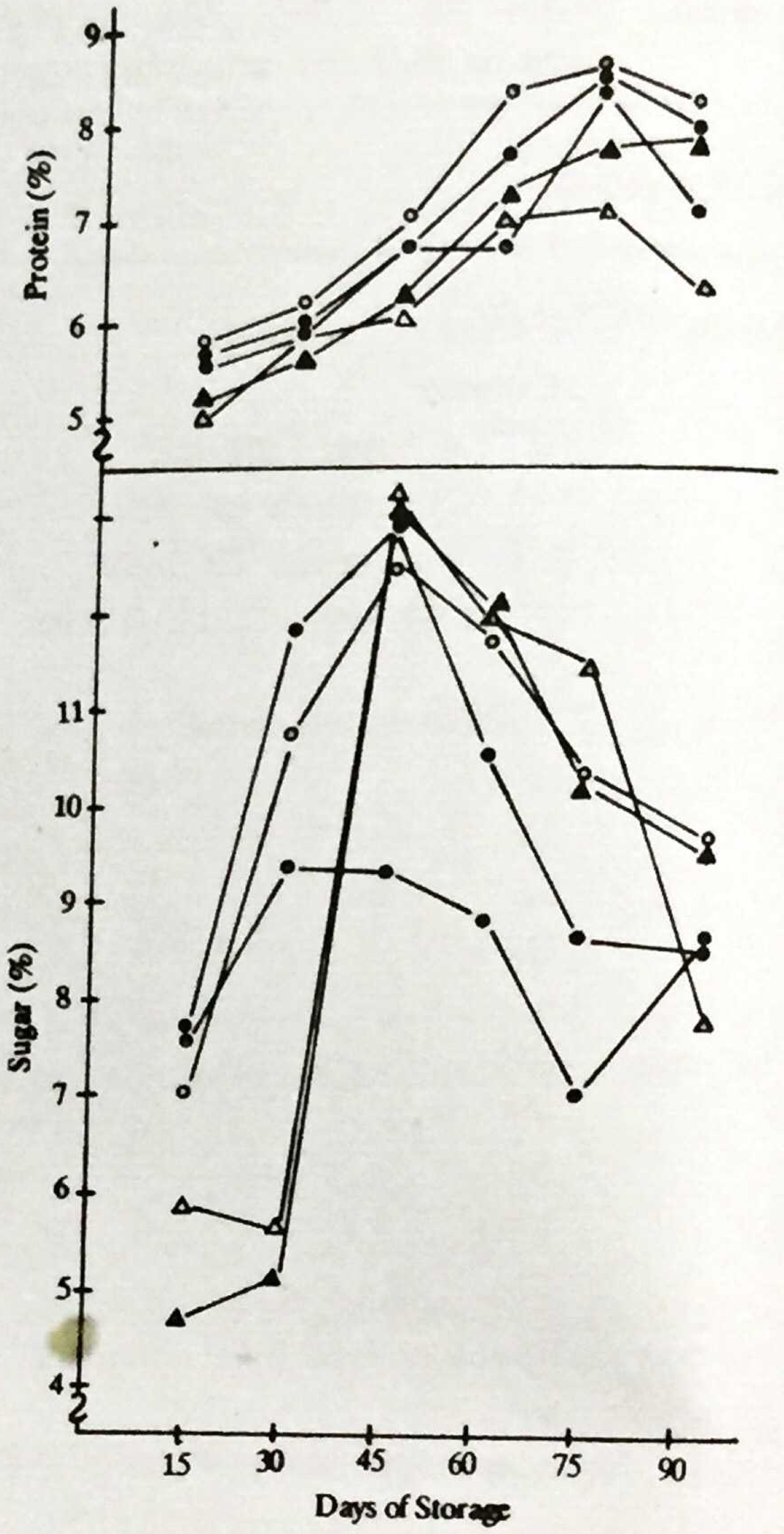
phoenicis produced relatively low amount of protein during the entire fermentation period. Using *A. phoenicis*, the amount decreased from 45 to 90 days of fermentation.

Fermentation from 15 to 30 days produced simple sugars which were used by the microorganisms as energy source for their growth and activity. As the amount of sugar decreased, the protein content of most isolates increased which meant that *A. sojae*, *A. oryzae* and *A. flavus* could consume the available sugars to produce reasonable amount of protein. Less protein was produced by *A. niger* and *A. phoenicis* in spite of the high amount of sugar they produced. These microorganisms possibly produced other products in amounts higher than the protein produced during fermentation. Furthermore, they consumed relatively less of simple sugars than *A. sojae, A. oryzae* and *A. flavus* except *A. phoenicis* where sugar decreased abruptly from 60 to 90 days of storage. In some cases, however, *A. niger* and *A. phoenicis* effect greater starch degradation than *A. sojae, A. oryzae* and *A. flavus* but all these microoganisms possibly use the same amount of simple sugars for their growth and activity.



Legend: O - *A. sojae*
ʘ - *A. oryzae*
● - *A. flavus* Link var. *columnaris* var. nov.
△ - *A. niger* van Tieghem
▲ - *A. phoenicis* (Cda.) Thom.

Figure 1. Amount of protein and sugar (%) produced by the microorganisms from 0 to 90 days of fermentation using sweet potato.



Legend: O - *A. sojae*
ʘ - *A. oryzae*
● - *A. flavus* Link var. *columnaris* var. nov.
△ - *A. niger* van Tieghem
▲ - *A. phoenicis* (Cda.) Thom.

Figure 2. Amount of protein and sugar (%) produced by the microorganisms from 0 to 90 days of fermentation using cassava.

With cassava flour, the highest amount of protein was produced by *A. sojae* at 60 to 90 days of fermentation but these amounts were comparable with those produced by *A. flavus* within 75 to 90 days and *A. phoenicis* at 90 days of fermentation (Fig. 2). *A. niger* generally produced the lowest amount of protein.

Table 6. Sensory qualities of sweet potato-based soy sauce using different types of mold inocula.[1]

Microorganism	Code	Appearance	Aroma	Color	Flavor	General Acceptability
A. sojae[2]		7.42a	6.48a	7.28a	7.20a	7.18a
A. oryzae[2]		7.45a	6.85a	7.42a	7.02ab	7.37a
A. flavus Link var. *columnaris* var. nov.	RT	7.30a	6.68a	7.58a	6.65ab	6.94ab
A. niger van Tieghem	LL	6.15b	6.20ab	6.02b	6.25bc	6.39bc
A. phoenicis (Cda.) Thom.	JJ	6.22b	6.52b	6.58b	6.75c	6.00c
C. V. (%)		6.91	6.34	6.82	6.58	6.78

[1]Means followed by a common letter are not significantly different at 5% level, DMRT.
[2]Obtained from Japan.

When *A. niger* and *A. phoenicis* were used, sugar content drastically increased from 30 to 45 days of fermentation. *A. flavus* produced high amount of both sugar and protein from 15 to 45 days of fermentation. This result is quite remarkable because molasses is usually added to enhance soy sauce taste and color. *A. niger* and *A. phoenicis* therefore could possibly lower cost by reducing the amount of molasses in soy sauce. The black pigments produced by these isolates contribute to the dark color of the soy sauce which means that this could replace molasses to darken soy sauce.

Sensory Evaluation of Root Crop-Based Soy Sauce

The acceptability of root crop-based soy sauce produced using different microorganisms differs in terms of sensory qualities. Using sweet potato flour, *A. flavus* produced soy sauce with appearance, aroma, color, flavor and general acceptability comparable to the control (Table 6). Although soy sauce produced by *A. niger* and *A. phoenicis* was still acceptable, their sensory scores were lower than that of *A. flavus* and the control. *A. flavus* and *A. niger* had the same scores in terms of general acceptability. Aroma of soy sauce produced by *A. niger* was also comparable with that of the control and *A. flavus*.

In cassava, the sensory qualities of soy sauce produced by *A. flavus* was similar with the control except for color. Only soy sauce produced by *A. oryzae* and *A. flavus* was comparable in color (Table 7). All isolates and the control produced soy sauce with undistinguishable difference in aroma. Appearance, flavor and general acceptability of soy sauce produced by

Table 7. Sensory qualities of cassava-based soy sauce using different types of mold inocula.[1]

Microorganism	Code	Appearance	Aroma	Color	Flavor	General Acceptability
A. sojae[2]		7.38a	6.40a	7.45a	6.68a	7.04a
A. oryzae[2]		6.78ab	6.50a	6.82ab	6.55ab	6.62ab
A. flavus Link var. *columnaris* var. nov.	RT	6.58ab	6.32a	6.55b	6.40ab	6.56ab
A. niger van Tieghem	LL	6.82ab	6.20a	6.72ab	5.68b	6.28b
A. phoenicis (Cda.) Thom.	JJ	6.10b	5.75a	5.80c	6.32ab	6.13b
C. V. (%)		17.66	19.77	17.80	20.66	16.05

[1]Means followed by a common letter are not significantly different at 5% level, DMRT.
[2]Obtained from Japan.

all isolates were the same as that produced by *A. oryzae* but not with that produced by *A. sojae*. Soy sauce produced by *A. phoenicis* had the least acceptable color.

Identification of Promising Isolates for Soy Sauce Production

The high protein-producing fungal isolates coded as RT, JJ and LL were identified as *Aspergillus flavus* Link var. *columnaris* var. nov., *A. niger* van Tieghem and *A. phoenicis* (Cda.) Thom., respectively (Raper and Fennel, 1965; Rayner, 1970). Macroscopically, *A. flavus* var. *columnaris* had a characteristic quite similar to *A. sojae* and *A. oryzae* when grown in CZD and MEA. They are all green except that *A. flavus* var. *columnaris* did not change to light brownish green with age. This behavioral growth of these mold species was also observed by Quimio (1982). *A. niger* and *A. phoenicis* are both velvety black in CZD and MEA. Unless microscopic examination is done, it is hard to distinguish one from the other. Microscopically, the conidial heads of *A. niger* in CZD is black while those of *A. phoenicis* is brownish black. This difference in color is undistinguishable macroscopically due to heavy sporulation which oftentimes makes the colonies appear carbon black.

Qualitative Analysis for Aflatoxin Formation

Results of qualitative analysis for aflatoxin formation in soy sauce produced by the test microorganisms showed negative results using either sweet potato or cassava flour. Not all strains of *A. flavus* therefore produced aflatoxin. Sajise (1985) reported that aflatoxin was not detected when *A. flavus* was artificially inoculated to processed cassava chips. Aflatoxin formation, however, is greatly affected by several factors. Hence, aflatoxin should be determined at varied conditions and substrates to ensure safety especially if the product is intended for animal or human consumption. Therefore; under soy sauce fermentation conditions, *A. flavus* is probably safe because it does not produce aflatoxin.

LITERATURE CITED

DATA, E.S., GALVEZ, F.C. and FORIO, E.E. 1983. Soy sauce production utilizing root crop flour as substitute for wheat flour (100% substitution). Terminal Report. PRCRTC-funded project on Post-harvest Technology of Root Crops under Philippine condition.

FRAZIER, W.C. 1967. Food Microbiology. McGraw-Hill Book Company, New York. pp. 409-410.

GALVEZ, F.C.F. 1980. Root crops flour: Successful substitutes for wheat flour in soy sauce production. Radix 2(2): 1, 9-10.

JOSON, L. 1978. Research on industrial microbiology in the Philippines. Paper presented in a Symposium on Microbiological Research and Technology. Philippine Society for Microbiology, Nov. 3, 1978, Philamlife Auditorium, Mla.

MENDOZA, J.D. 1961. Philippine Foods: Their processing and manufacture. McCullough Printing Co., PEC. Mla. pp. 186- 189.

RAPER, K.B. and FENNEL, D.I. 1965. The genus *Aspergillus.* The Williams and Wilkens Company, Baltimore.

RAYNER, K.W. 1970. A mycological chart. Commonwealth Mycological Institute and British Mycological Society. Surrey.

SAJISE, C.E. 1985. Post production aflatoxin contamination in cassava (*Manihot esculenta* Crantz) in the Philippines. Unpublished MS Thesis. UPLB, College, Laguna, Philippines.