MICROBIOLOGICAL STUDIES OF GUINEA CORN FERMENTATION FOR OGI-BABA PRODUCTION.

BY

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ABSTRACT

The microbiological and biochemical aspects of ogi-baba production from sorghum were investigated. The microorganisms on the unfermented grains, the steeping and souring water were isolated. The unfermented grains contained Bacillus subtilis, Lactobacillus plantarum, Streptococcus lactis, Aspergillus sp. and Penicillium sp. The microorganisms found during steeping were L. plantarum, B. subtilis, S. lactis, Pediococcus sp., Leuconostoc sp. and Debaryomyces hansenii. At souring, S. lactis, L. plantarum and Candida krusei were isolated.

The predominant microorganisms during the fermentation were L. plantarum, S. lactis, D. hansenii and C. krusei.

The pH during fermentation decreased from an initial value of 6.4 to 3.8 with substantial titratable acidity.

Pure cultures of L. plantarum, S. lactis, D. hansenii and C. krusei individually fermented the sorghum flour poorly when compared to the combined effect of L. plantarum, S. lactis and D. hansenii. The sample inoculated with 2% by steep liquor produced ogi-baba similar to the locally fermented ogi-baba.

The physiological studies carried out on the isolates showed that optimal growth temperatures were 30°C for L. plantarum and S. lactis on one hand and 40°C for C. krusei. The pH optimal for

growth were 4.5, 5.0 and 6.0 for the two Lactobacillus sp. and S. lactis respectively. It was pH 4 and 5 for D. hansenii and C. krusei respectively.

Qualitative determination of sugars by thin layer and paper chromatography showed that the unfermented grains contained raffinose, maltose, sucrose, glucose and fructose. Raffinose and maltose were in trace amounts. Sucrose and raffinose disappeared by 24 hr and at souring, only glucose and traces of maltose were present.

Alpha-amylase activity was not detected in the unfermented grains. Invertase and alpha glucosidase were present in the grains with the peak of activity at 36 hr of steeping. Debaryomyces hansenii contained the highest invertase and alpha-glucosidase activity. Trace amounts of these enzymes were observed in L. plantarum and S. lactis.

The total ethanol soluble sugars increased as starch decreased.

Analysis of sorghum processing waste for ogi-baba showed that it contained 28.8% carbohydrate, 8.18% protein, 18.0% fat and 1.5% total ash. The utilization of this waste for producing amylase enzyme by B. subtilis was optimal at 72 hr, 30^{o}C and pH 6.5 but requires the addition of 1% urea.

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C E R T I F I C A T I O N

I hereby certify that this work was carried out by Miss Senapon Adeyele under my supervision in the Department of Botany and Microbiology, University of Ibadan, Nigeria.

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D E D I C A T I O N

This work is dedicated to the memory of my late father Chief M. O. Adeyele and my entire family especially my mother Bisi Adeyele for her moral and spiritual support after the death of my father and also my brothers and sisters (from brother Agba to little Penny) in their little ways.

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CHAPTER 1

INTRODUCTION

In most developing countries, fermented foods and beverages constitute a significant component of the diet. These fermented foods which serve as main meals are usually from fermentation of carbohydrate-rich raw materials, most of which have low protein and vitamin content. Some of the most important ones in Nigeria include ogi from maize, ogi-baba from sorghum, fufu and gari from cassava.

The common substrates for fermentation in Tropical Africa are cassava, different types of beans and cereal grains, such as maize, sorghum and millet. The cereal grains are fermented and milled to produce thin gruel and alcoholic beverages which are known by various names in different parts of West Africa (Odunfa, 1985). In Yorubaland of Nigeria, maize gruel is known as ogi, sorghum gruel is ogi-baba. If the ogi is cooked to produce a semi-solid product, it is called agidi in Ibo or eko in Yoruba.

Ogi differs from other African cereal beverages such as mahewu (of the Bantus of South Africa), uji (a maize beverage in East Africa) and akasa (a maize beverage in Ghana).

These are farinaceous and gritty unlike ogi which is a sour fine paste.

Of the cereals which are consumed, Guinea-corn is one of the most important; 75% of the total calories from cereals in Zaria Province in Nigeria is from Guinea corn. As a source of calories, it was found to be the third cheapest after millet and tuwo of 26 foods compared (Simmons, 1976). Survey in three Zaria villages in Nigeria showed that cereals constitute 1,588 calories out of a total of 22,641 daily per capita calories intake.

Ogi-baba is one of the most important fermented cereals in West Africa. This is so since sorghum is the most widely cultivated cereal in Nigeria and other West African countries. The Federal Office of Statistics, Nigeria (1983) estimated that about 2.79 million tons were produced in 1979/80 season from parts of Northern Nigeria.

Ogi-baba is widely used as food for weaning infants and as food for pre-school or school children and adults. It is a popular breakfast meal in Nigeria with 87.9% of it being home produced (Simmons, 1976). Nutritionally, cereals are deficient in cystine, tryptophan and in methionine (Pickett, 1966; Adeniji and Porter, 1978) and low in essential amino acids. The deficiency in protein is even higher after processing (Jacques, 1970).

To off-set this deficiency in protein in ogi, a protein rich soy-ogi was developed (Akinrele et al, 1970) by incorporating soya beans into the ogi during fermentation.

The chemical compositions of maize (Zea mays Linn) and sorghum (Sorghum guineense or bicolor) are similar (Table 1). However, there are a few significant differences, sorghum contain more protein than maize. In addition, sorghum contains phenolic constituents including tannins and these generally have inhibition effects on microbial growth and may consequently affect the selection of microorganisms in the fermentation (Watson, 1975). Table 1 shows that the protein content of cereal grains averages 10%. Nuecere and Sunrell (1980) compared the chemical composition of different varieties of sorghum grain and reported them as having 9.75 - 14.32% protein, 2.66 - 3.49% fat, 67.61 - 73.37% carbohydrate and 1.35 - 1.83% ash expressed as percentage of whole seed.

Fermentation of sorghum produces the pleasant flavour enjoyed by ogi-baba consumers and also improves the nutritive value (Van Veen and Steinkraus (1970); Hamad and Fields (1979) and Tongnual and Fields (1979)). Tongnual and Fields (1979) and Hamad and Fields (1979), working on fermented whole maize, millet, rice and wheat, reported an increase in relative nutritive value including significant increase in lysine content in fermented

TABLE 1. Chemical composition of maize and sorghum.

g/100g edible portion

Product	Protein	Fat	Carbohydrate	Sugar	Ash
Whole maize	9.3[a]	4.8[a]	73.7[a]	1.49[a]	1.4[b]
Sorghum	11.1[a]	3.2[a]	74.1[a]	-	-
	(11.0)[d]	(3.3)[d]	(73.0)[d]	2.0-4.5[c]	1.7[d]
	(9.17)[e]	(3.25)[e]	(76.18)[e]	-	(2.36)[e]

a = Latham (1979).

b = Casey & Lorenz (1977).

c = Subramanian et al., (1980).

d = Watt et al., (1975).

e = Oyenuga (1968).

rice, millet and maize. Andah and Muller (1973) reported increases in thiamine content of fermented Ghanaian maize porridge, while Cameron and Hofvander (1971) reported an increase in riboflavin and niacin content in whole fermented grain in West Africa.

Raw foods generally harbour miscellaneous micro-organisms, mostly aerobic and facultative. Grains and seeds harbour microorganisms of the air and soil origin. During studies on storage of corn, McMahon et al. (1975) observed aerobic mesophiles and coliforms during the early stages of storage but later the population decreased. The majority of organisms on vegetables and cereal products are aerobic and facultative types e.g. Pseudomonas, Flavobacterium, Escherichia and Bacillus but relatively few species are known to be responsible for fermentation (Pederson, 1979).

Various workers have carried out studies on fermentation of maize for ogi. Akinrele and Bassir (1967) isolated some of the microorganisms of ogi and pointed out that Lactobacillus plantarum was the predominant microorganism at the souring stage. Akinrele (1966, 1970) carried out intensive studies on the fermentation and biochemical changes during the production of ogi from maize. He contended that steeping of maize in water brings about the natural selection of desirable micro-organisms. He further

observed that the fungi - Cephalosporium sp., Fusarium sp., Aspergillus sp. and Penicillium spp. were eliminated during the early steeping stage. Akinrele (1970) reported that Aerobacter cloacae, Corynebacterium michiganense, Leuconostoc mesenteroides, Lactobacillus plantarum and Candida mycoderma were involved in the fermentation. Hesseltine (1979) in his review reported that Streptococcus lactis was isolated as the main microorganism in mahewu (a South African beverage similar to ogi) and that the acid is unstably formed.

Lactic acid bacterial fermentation during commercial corn steeping was observed by Watson et al. (1955). Organisms obtained were L. plantarum and S. lactis. Banigo and Muller (1972) compared the ogi making properties of maize, sorghum and millet and concluded that both maize and sorghum have better yields than millet.

Vander Walt (1955) isolated Lactobacillus fermenti, Streptobacterium sp., Lactobacillus brevis, Leuconostoc mesenteroides and Leuconostoc dextranicum from puring mash of kaffir corn during kaffir beer production. Ekundayo (1969) investigated the fermentation of sorghum for pito production and isolated Aspergillus flavus, Rhizopus oryzae, Penicillium citrinum, Candida sp. and Lactobacillus sp. Faparusi et al. (1973) isolated Saccharomyces sp., Candida sp., Aspergillus sp. and Penicillium sp. during the

investigation of fermentation of a mixture of sorghum and gari for burukutu. They also observed that the two bacterial types found in the malts were Lactobacillus and Streptococcus spp. In other vegetable and cereal-based fermented products, L. mesenteroides and Steptococcus species have been reported to initiate fermentation followed by homo-fermentative L. buchneri, L. brevis and finally by homo-fermentative Pediococci and L. plantarum (Pederson and Albury,(1950, 1956), Christensen et al., (1958) and Stammer et al.,(1971).

In order to develop starter cultures for fermentation, information is needed on the specific microorganisms involved in the fermentation. There have been few attempts along this line. Such studies include those of Christian (1970) in Ghana, working on maize dough, who found that the most important bacteria for the fermentation are Lactobacillus fermenti, Leuconostoc mesenteroides and Pediococcus cerevisae. Onyekwere and Akinrele (1977) in Nigeria produced an acceptable ogi using a 24 hr steep liquor and a combination of Lactobacillus, Aerobacter and Saccharomyces species.

Starter cultures have been developed for the production of busaa, a Kenyan opaque maize beer (Nout, 1980), uji, an East African sour cereal porridge (Mbugua, 1981) and tempeh, an Indonesian food (Hesseltine, 1963). Studies with strains of L. delbrueckii,

L. bulgaricus, L. acidophillus and S. lactis as starters for mahewu fermentation, a South African sour maize beverage, showed that L. delbrueckii was most efficient in acid production (Schweigart and Fellingham, 1963). The symbiotic relationship of S. thermophillus and L. bulgaricus has been utilized in mixed cultures for yogurt production (Bautista et al., 1966; Veringa et al., 1968; Moon and Reingold, 1976).

In South East Asia, China and India, starter cultures are widely used and are called such local names as murcha in China, ragi in India (Hesseltine, 1983) and koji in Japan.

Two of the problems associated with traditionally fermented ogi-baba are off-flavour and irregularity of flavour. These might be due to the presence of some undesirable micro-organisms and the varied conditions under which the ogi-baba is produced. Although the contaminants might also include pathogens, these do not pose much problem as the product is usually well cooked before consumption. Moreover, the acidic nature of the fermented product makes it unfavourable for the growth and activity of pathogenic and spoilage organisms (Fulde and Fabian, 1953; Van Veen and Steinkraus, 1970; Frank and Marth, 1977). Also changes in physical factors can affect the fermentation. For example, the fermentation of mahewu, a South African sour beverage proceeds faster at 45^{o}C (Hesseltine, 1979).

During cereal fermentation, the starch is converted to sugars, then to acids by either the cereal or microbial enzyme activity. Information shows that the saccharification of the grain starches may be due to fungal amylases; for example, during malting of kaffir beer (Platt, 1964). Nout (1980) is of the opinion that the cereal amylases are the effective agents of saccharification. Wodzinskii (1979) reported extra-cellular enzymes especially hydrolases to be produced by Lactobacillus and Bacillus spp. which are involved in fermentation processes. Mital et al. (1973) reported the presence of alpha-galactosidase in L. fermenti, L. brevis, L. cellobiosus, L. buchneri and L. salivarus. Mizokami et al., (1977) reported extra cellular amylase in Streptococcus bovis which fermented raw starch. On the other hand, Treit (1967) observed Candida utilis and Endomycopsis fibuliger to be amongst the yeasts that utilise starch present in fermentation processes because of their amylolytic properties.

Sugars will play an important role in the fermentation of starchy foods such as cereals. It serves as the easily available metabolizable substrate for the growth of microorganisms. It may also be one of the factors imparting taste and flavour to food products (Subramanian et al., 1980) as well as acting as the substrate for lactic acid. The lactic acid produced is the main

flavour compound in acidic cereal beverages such as ogi, uji and mahewu (Akinrele, 1966; Mbuqua, 1981).

During the processing of sorghum for ogi-baba production, considerable waste is produced. This waste contains the pericarp, germ as well as some residual starch. It is estimated that the pomace constitutes about 30% of the original weight of guinea corn used in the production. The waste generated from guinea corn is higher than that generated by the same weight of maize for ogi production. Traditionally, this waste is discarded or may be used for poultry reared around the compound. In future it is anticipated that ogi-baba will be produced on an industrial scale; hence the processing waste will pile up to huge levels and may constitute a disposal problem. The trend in agro-industrial development is to have an integrated approach, that is a system where the by-products of the industry are utilized.

Since the processing waste contains a high proportion of starch, the waste would serve as a useful substrate for the production of amylase. Amylase is an important industrial enzyme. It is used in the preparation of adhesives, in desizing (removing starch) in textile industries. In the brewing industry, it may be employed as a replacement for malt for releasing additional fermentable sugars. Perhaps the biggest application of amylases is the bioconversion of starchy materials to dextrose (Underkofler, 1976).

Agro-industrial by-product such as sorghum waste is in fact most suitable for many fermentation industries because of its low economic value.

In order to produce this amylase, it is necessary to obtain the appropriate amylase-producing microorganism. Secondly, since the waste will mainly provide the source of carbon, other nutrients such as salts, nitrogen source etc. need to be added to develop a suitable fermentation broth. Thirdly, the optimum conditions such as pH, temp etc. for optimum enzyme production need to be investigated.

In most of the earlier work, information is available only on the microorganisms associated with the fermentation process. Less attention was given to investigating the organisms actually involved in the fermentation; hence the role of each culture in the fermentation was hardly appreciated. Moreover, research in fermented gruels in West Africa has so far been limited to maize ogi and no information is available on guinea corn fermentation.

The objectives of this investigation are therefore as follows:

1. To investigate microbial and chemical changes during traditional fermentation of sorghum for ogi-baba production;
2. To examine the carbohydrate changes and their utilization by micro-organisms during the fermentation;
3. To assay for the enzyme activities during the fermentation;

4. To optimize the fermentation process through development of pure culture fermentation;
5. To formulate a medium for utilizing processing waste for the production of industrial enzymes, specifically amylases.

CHAPTER 2

MATERIALS AND METHODS

(a) Microbiological analysis during preparation of ogi-baba

The traditional method of preparation of sorghum ogi was followed in which sorghum is soaked for 1 - 3 days and the steep liquor decanted off. The steeped grains were then wet-milled and sieved to remove the pomace which was discarded. The filtrate was allowed to settle and become sour. The process is illustrated in Fig. 1 and Plate 1.

(b) Culture media

Different media were used for the preliminary isolation. This was aimed at selecting the various micro-organisms occurring during the steeping and souring stages of sorghum fermentation. The media used were malt agar (MA) for yeasts and moulds; potato dextrose - streptomycin agar (PDA) for fungi; APT agar (Evans and Niven, 1957) and MRS agar (de Man et al., 1960) for lactic acid bacteria and Standard plate count agar (PCA) for total viable count. All the media were oxoid (Basingstoke, U.K.) formulations. For subsequent work and subculturing, MRS agar was used for lactic acid bacteria and MA (Malt agar) was used for yeasts and moulds.

(c) Isolation of microorganisms

Steep liquor (10ml portions) was aseptically removed each day

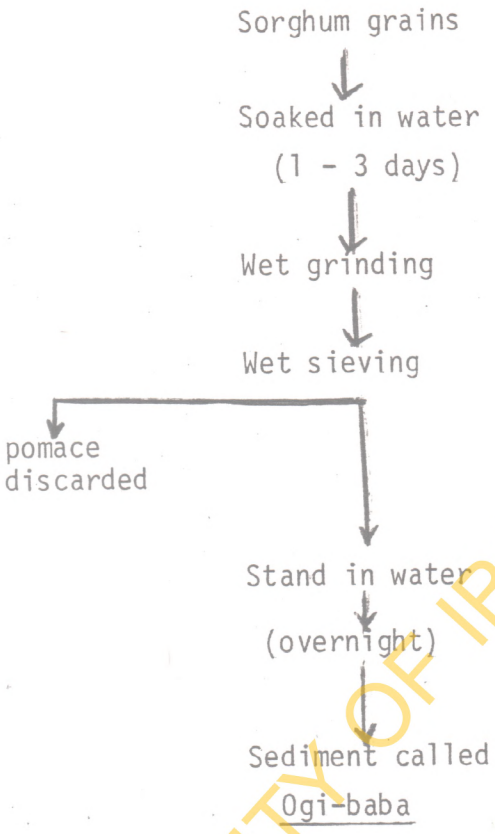


Fig. 1. Flow chart of process for producing ogi-baba in the traditional way.

Plate 1. A Yoruba woman preparing ogi-baba in the traditional way; this is the sieving stage of the production.

and serially diluted (Taylor, 1962). The 10^{-6} and 10^{-7} dilutions were plated out by mixing with different media in McCartney bottles and poured aseptically into sterile petri dishes. Half of the places were incubated aerobically at 30°C while the other half were incubated at 30°C in an anaerobic gas jar. The anaerobic atmosphere was provided by using BBL hydrogen and carbon dioxide generators.

After 24 and then 48 hr, the plates were examined and the colonies counted. Representative colonies were streaked out to obtain pure cultures. Each pure culture was then inoculated onto slants and kept as the stock culture; MRS (de Man et al., 1960) for the lactics and MA (Malt agar) for the yeasts. They were subcultured every 4 weeks.

(d) Physicochemical changes

Twenty-five grams of the sorghum grains were weighed and put in a beaker containing 500ml water and left on the laboratory bench to ferment. The oxygen depletion during the fermentation was determined using a Biological oxygen monitor YSI model 53 (Yellow Springs Instrument). The electrode was dipped inside the fermenting broth. At 24 hourly intervals, the percentage saturation was recorded.

(e) pH measurement

The pH changes of the steep liquor were measured immediately

and at 12 hourly intervals for 4 days using Pye-Unicam pH meter, model 291 MK2 equipped with a glass electrode.

(f) Titratable acidity

The aim is to estimate the relative amount of lactic acid present in ogi-baba during fermentation and the titratable acid is expressed as % LA as a guide to the assessment of ogi-baba taste.

The method adopted was that used by Akinrele and Oguntade (1976). This involves titrating 10ml of steep liquor containing 10ml of distilled water and 5 drops of 1% phenolphthalein solution (as indicator) against 0.01N NaOH till a permanent faint pink colour was obtained. The acidity was calculated as % lactic acid according to the following formula (Frazier et al., 1968).

$$\% \text{ Lactic acid} = \frac{\text{ml alkali} \times \text{normalty alkali}}{\text{wt of sample in g}} \times 9$$

assume 1ml = 1g

(g) Characterization of isolates

Characterization of isolates was carried out by employing microscopic, physiological and biochemical tests.

The cultures isolated were Gram stained according to the method of Pelczar and Chan (1977), tested for catalase production by addition of a loopful of culture to a drop of hydrogen peroxide on a clean microscope slide and the motility was determined using hanging drop method (Seeley and Vandemark, 1972). The cell

morphology was examined using 24 hour-old culture.

(i) Oxygen relationship

Yeast extract agar (semi-solid 10ml portion) was dispensed into screw-capped tubes and sterilized. Each isolate was inoculated by stabbing with a needle down the length of the tube. The tube was then incubated at 30°C for 5 days.

When growth occurs at the surface, the organism was assumed to be aerobic; when growth occurs throughout the length of the tube, it is assumed to be faculative anaerobe and when growth is near the surface, it is micro-aerophilic and when at the bottom it is anaerobic.

(ii) Methyl Red-Voges-Proskaeur Test

Oxoid MR-VP (dehydrated medium) was prepared in culture tubes and autoclaved. After autoclaving, 2 tubes were inoculated with each isolate and incubated at 30°C.

After 5 days, 5 drops of methyl red solution (0.2% ethanolic solution) were added to one tube. A red colour indicating pH 4.5 or less is positive for methyl red test. It shows high acid production.

To 1ml of culture of other tube, 6% α-naphthol and 16% NaOH were added and mixture shaken well.

Development of red colour within 5 min is positive. Positivity shows organism produces acetylmethylcarbinol from glucose.

(iii) Starch hydrolysis - Nutrient agar (5.6g) was dissolved in 150ml of distilled water. Soluble starch (0.4g) was separately prepared in 50ml of water by suspending the starch in water and using steam to dissolve. This was then mixed with Nutrient agar to give a 0.2% soluble starch Nutrient agar medium. After sterilization at 121^{o}C for 15 min, the agar was poured into sterile Petri dishes. Each plate was streaked across once with each isolate. The plates were incubated at 30^{o}C for 4 days, after which the plates were flooded with Grams iodine. Absence of bluish-purple colour indicates hydrolysis and is as a result of the presence of enzyme amylase.

(iv) Fermentation of sugars

The basal medium used was phenol red in peptone water (Oxoid). Nine ml of this was dispensed into each screw-capped test-tube into which a Durham tube has been previously inserted; the tubes were subsequently sterilized by autoclaving at 121^{o}C for 15 minutes.

The sugars (glucose, raffinose, mannitol, mannose, sucrose, fructose) were prepared as 10% solutions and sterilized separately.

Glucose and fructose were filter sterilized. The sugars were then added to the basal medium to give a final concentration of 1% fermentable sugar. The tubes were inoculated with each isolate. An uninoculated tube served as the control. These were incubated at 30°C for 4 days. A change in colour to yellow indicates acid production. Proteolysis was assessed by using litmus milk, and casein hydrolysis (Conn et al., 1957).

(v) Reduction of nitrate

Nitrate is reduced by some micro-organisms to nitrite, ammonia or free nitrogen.

Nitrate peptone broth containing 0.2% KNO_3, 1g of peptone and 0.5g NaCl dissolved in 100ml of distilled water was dispensed into tubes. Each tube contains an inverted Durham tube to detect gas production. The tubes were then sterilized. After cooling, the isolates were inoculated into the tubes and incubated at 30°C for 4 days. The presence of nitrite was tested for by adding a few drops of

dimethyl naphthylamine to each tube. A red colour developed in positive tubes. Presence of gas in Durham tubes indicates production of nitrogen gas.

(vi) Production of gas from glucose

This is a test used to differentiate between homo - and hetero-fermentative lactic acid bacteria.

Gibson's semi solid medium (Gibson and Abdel-Malek, 1945; Stammer et al., 1964) composed of the following:

Yeast extract	2.5g
Glucose	50g
$MnSO_4.7H_2O$	0.04g/10ml distilled water.
Reconstrituted skim milk	80g/800ml distilled water.
Nutrient agar	5.6g/190ml distilled water.

The manganese sulphate solution was mixed with the reconstituted skim milk. The yeast extract and glucose were added, the mixture was heated in the steamer and molten NA was added to the hot mixture from the steamer and mixed. The pH was adjusted to 6.5. The mixture was distributed into test-tubes to a depth of 5 - 6cm and sterilized by steaming for 30 min on each of 3 successive days. At the end of

the third day when the mixture was cool, it was inoculated with 0.5ml portion of young MRS broth culture of isolated bacteria, mixed and cooled in tap water to solidify. Then molten Nutrient agar was poured into each tube at about 50°C giving a layer 2 - 3cm deep above the surface of the medium. When agar seals are set, the tubes were incubated at 30°C for 14 days and observed every 48 hours.

(vii) Hydrolysis of Sodium hippurate

Organisms were grown for 14 days in MRS broth containing 1% w/v Na hippurate (Naylor and Sharpe, 1958). Then 5ml of MRS broth culture were added to 1.5ml of 50% H_2SO_4. Formation of white crystals of benzoic acid indicates positive reaction. The mixture of 50% H_2SO_4 and supernatant were warm when mixed together and hence it was allowed to stand for 30 - 60 min before results were taken.

(viii) Production of ammonia from arginine

Nivey et al. (1942) reported this method of detecting production of ammonia from arginine by Streptococci. In this test, 0.3% (w/v) L-arginine monohydrochloride was added to MRS broth before

autoclaving. The same medium without arginine served as the control. Nessler's reagent was used to test for the presence of ammonia after 14 days incubation.

(ix) Growth temperature experiments

The growth of the cultures at 45°C and 15°C was used as a differential test. To 0.5ml of 24-hour-old broth culture was added 20ml of MRS broth. The cultures were then incubated at 15°C and 45°C for 7 days. Turbidity of the MRS broth was used to indicate growth.

(x) Identification of micro-organisms

Identification of bacterial species was on the basis of their Grams reaction and biochemical tests and by reference to Bergeys manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). Simplified schemes for identification of yeast cultures (Beech et al., 1968) and identification scheme for the lactic acid bacteria (Sharpe, 1979).

The identification of yeasts was confirmed by the National Collection of Yeasts Cultures, Norwich, England.

(h) Physiological studies on the dominant isolates

Aspects of the physiology of the following dominant isolates in the fermentation mixture were studied. The organisms were:

Streptococcus lactis, *Lactobacillus plantarum*

Debaryomyces hansenii and

Candida krusei

(i) Effect of temperature on growth

The effect of temperature on the growth of the pure isolates was determined. Twenty ml of sterilized MRS broth was dispensed into 100ml flasks and was inoculated with the different isolates. The flasks were incubated at 25, 30, 35, 40, 45, 50 and 60^{o}C. Five millilitres of the culture medium were mixed for 5 sec using a Vortex mixer. Growth was assessed every 6 hours for 48 hr by measuring the turbidity at 540nm using an SP6-250 Pye Unicam Spectrophotometer.

(ii) Effect of pH on growth

A double strength solution of MRS broth (for the lactics) and Malt Extract broth (for the yeasts) were prepared and 15ml dispensed into 150ml flasks. Acetate buffer of 0.2M was prepared using 0.1M citric acid and 0.2M sodium dihydrogen phosphate (Hales, 1958).

The pH of the broth in each flask was adjusted to the desired pH ranging from 2 - 8 by adding 15ml of appropriate buffer (v/v) to each flask. This made the MRS and MA broth single strength. In adjusting to the required pH values, a few drops of 0.1N HCl were added. The pH of the broth after autoclaving was checked by aseptically taking 5ml of the broth and measuring the pH. The flasks were inoculated and incubated at 30°C for 48 hours.

Bacterial growth was determined colorimetrically at 540nm while the growth of the yeasts was obtained by the dry weight method.

(i) Biochemical analysis during fermentation

Preparation of extracts for chromatographic analysis of sugar in the fermenting mash.

The different fermenting samples at 0, 24, 48 and 72 hr of steeping and souring were extracted by refluxing 0.5g of dried sample with 25ml of 80% ethanol for 1 hr. The proteins and tannins were precipitated with 0.5ml of 1% basic lead acetate in water and the extract was then centrifuged at 8,000g for 10 min using 'Universal' table-top centrifuge.

(j) Sugar analysis by paper chromatography

Aliquots of 25 µl of the extract from the different samples and a mixture of known sugars (as reference) were spotted on Whatman No. 4 chromatography paper. The reference sugar solution contained 0.1% of glucose, fructose, maltose, raffinose, xylose and sucrose. The chromatogram was developed for 12 hr in butanol-acetic acid-water (4:1:1) using descending paper chromatography method. After drying in the air, the sugars were detected following the method of Trevelyan et al. (1950). The sugars appeared as black or dark brown spots against a white background.

(k) Analysis of oligosaccharide by thin layer chromatography

Aliquots of 50 ul of the extract were spotted on 'Avicel' micro-crystalline cellulose plates along with 25 µl of reference mixture containing 0.1% of stachyose, raffinose, sucrose, fructose. A one-dimensional ascending chromatography was done using a solvent system of n-propanol-ethyl acetate-water (6:1:3 v/v). After 6 hr, the oligosaccharides were located by spraying with α- naphthol in acetone (Albon and Gross, 1950). The plate was then dried for 15 min at 90°C.

(l) Assay of ethanol-soluble sugars

The quantities of ethanol-soluble sugars present in the fermenting grains and souring samples were determined quantitatively

by using phenol-sulphuric acid method (Dubois et al., 1956). Each powdered sample of 0.5g was extracted for 6 hr in a Soxhlet extractor with 25ml of boiling 80% ethanol. The extract was dried in a boiling water bath and the residue redissolved in 5ml distilled water.

One millilitre of 5% (v/v) phenol was added to 1ml of the extract. From the preliminary experiment, it was found necessary to dilute the extract appropriately to obtain good readings. Five ml of concentrated sulphuric acid from detipped burette was quickly added to the mixture which was allowed to cool. The optical density was read at 490nm using Pye Unicam SP6-250 Spectrophotometer.

The quantity of ethanol-soluble sugar was determined by reference to a standard curve based on known concentrations of glucose.

(m) Assay for starch

The residue after extraction of sugars was finely ground with mortar and pestle and sifted through a sieve of pore size 250um.

Starch determination was carried out by diastase hydrolysis following method of Shriner (1932) as modified by Barnell (1936).

Ten milligrams of residue from extraction of the total sugar was put in a McCartney bottle and 2ml distilled water was added.

The residue was heated in a boiling water bath for 30 min to gelatinize the starch, allowed to cool and then 2ml of freshly prepared 1% diastase (BDH Chemicals, England) was added. The mixture was incubated at 37°C for 24 hr. The blank contained 2ml distilled water and 2ml of 1% diastase.

After incubation, the mixture was heated to boiling to terminate the reaction and then it was filtered. The insoluble material was washed with hot water and filtered. The two filtrates were combined and cleared with 0.5ml of 1% basic lead acetate (Bacon and Edelman, 1951). The mixture was then diluted to 500ml with distilled water, shaken vigorously and the precipitate allowed to cool and the suspension filtered through Whatman No. 1 paper. Excess lead ions were removed with 2ml of absolute methanol. The blanks were treated similarly.

The hydrolysed starch was quantitatively determined using phenol-sulphuric acid method of Dubois et al. (1956) at 490nm.

In estimating the amount of starch from glucose curve, the weight of starch was multiplied by a 0.9 constant (Hassid and Nuefield, 1964).

(n) Sugar utilization

The cultures used in this study were Lactobacillus plantarum, Streptococcus lactis and the two yeasts. They were isolated from fermenting Sorghum and maintained on APT agar (Evans and Niven, 1957)

MRS agar (de Man Rogosa and Sharpe, 1960) and malt agar respectively.

The inoculum was grown in the broth form of each medium. The basal medium (50ml) was sterilized in 150ml flasks. The medium consisted of $NaNO_3$, 5g; $K_2H\ PO_4$, 1g; $MgSO_4.7H_2O$, 0.5g; $Ca(NO_3)_2.4H_2O$, 0.01g; $FeCl_3$, 0.001g; Yeast extract; 0.5g; distilled water, 1 litre. The following sugars xylose, glucose, fructose, lactose, galactose, maltose, sucrose and raffinose were sterilized at 1.05kg/cm^2 for 10 min except glucose that was filter sterilized. The sugars were subsequently added singly to the basal media to give 0.5% final concentration. The media were inoculated with 0.1ml of each culture and incubated at 30^{o}C for 48 hr.

(o) Assessment of microbial growth

At 12 hr intervals, 10ml samples of uniformly suspended culture were examined for growth by acid production. A Pye-Unicam pH meter model 291 MK2 equipped with a glass electrode was used to measure pH changes. Growth of the lactic acid bacteria was determined by the titratable acid produced. Titratable acidity was determined by measuring 5ml of the culture sample at each 12 hour and titrating with 0.025N NaOH with phenolphthalein as indicator. Titratable acidity was calculated by subtracting the amount of acid present in the uninoculated medium sample from that present in the test medium.

In case of the yeasts, the growth was determined by the dry

weight method.

(p) Preparation of enzyme extracts

(i) From sorghum grains

The grains were surface sterilized by washing with 1% Benomyl fungicide and were subsequently rinsed in three washings of sterile distilled water.

The grains were steeped in clean tap water for 0, 12, 24, 36, 48, 60 and 72 hr. Five grams of each sample were weighed and 0.02M phosphate buffer (25ml), pH 6.5 was added, ground with pestle and mortar, mixed thoroughly and filtered. The filtrate was then centrifuged at 25,000g in an MSE high speed 18 refrigerated centrifuge at $5^{o}C$ for 20 min. The enzyme extracts were then stored in the deep freezer until ready for use.

(ii) From microbial isolates

Sorghum extract was used since the microorganisms were isolated during fermentation of sorghum. Sorghum grains (50g) were ground and 250ml distilled water added and this was boiled for 30 min after which it was filtered. The filtrate was made up to 1 litre with distilled water and pH adjusted to 5.5 with 0.1N HCl. The extract was used as a basal medium by the addition

of glucose, 1g; NH_4Cl, 0.05g; KH_2PO_4, 0.15g; $MgSO_4 .7H_2O$, 0.05g. Then 25ml portion was distributed into 100ml flasks and sterilized at 1.05 kg/cm^2 for 15 min (Ekundayo, 1969).

The isolates were inoculated and incubated at 35°C for 4 days in a New Brunswick Gyrotary shaker at 250 r.p.m. At the end of 4 days, the medium was centrifuged using an MSE high speed refrigerated centrifuge at 22,000g at 5°C for 20 min. The supernatant was carefully decanted and kept in a deep freezer.

(q) Enzyme Assay

(i) Total amylase activity

One millilitre of enzyme extract was incubated with 1ml of 1% soluble starch in 0.02M phosphate buffer (pH 6.5) for 1 hr. The amount of reducing sugar released in the reaction mixture was estimated by adding 2ml of dinitrosalicylic acid (DNSA) reagent (Bernfield, 1951) and heated in a water bath for 5 min. Five ml of distilled water were added and the transmittance read at 540nm using Pye-Unicam SP6-250 Spectrophotometer. The amount of reducing sugar produced was determined from a standard curve prepared from maltose with concentrations ranging from 0.125 - 20mg per ml. The enzyme activity is

expressed as the amount of reducing sugar produced by 1ml of filtrate at 40°C for 1 hr.

(ii) Alpha-amylase activity

This was determined by heating the enzyme extract in a water bath at 70°C for 15 min to inactivate the β-amylase (Bernfield, 1951). Then 1ml of the heated extract was then incubated with 1ml of 1% soluble starch in buffer and treated as above.

(iii) Alpha-glucosidase activity

Five grams of different grains were extracted in 0.05M Tris buffer (pH 7.0) and the extract was centrifuged in an MSE high speed refrigerated centrifuge at 22,000g for 20 min at 5°C.

The α-glucosidase activity was determined by measuring the glucose end product using glucose-oxidase method (Hoggett and Nixon, 1957).

$$\text{Maltose} + H_2O \xrightarrow{\alpha\text{-glucosidase}} 2 \text{ glucose}$$

The enzyme extract (1ml) was added to a substrate sugar (1ml maltose) and incubated at 35°C for 1 hr. The resulting glucose was assayed by the glucose-oxidase reagent prepared by dissolving 2.0mg peroxidase and 20.0mg glucose-oxidase in 60ml Tris buffer and adding 1ml of O-Dianisidin (50mg dissolved in 10ml of 96%

ethanol). Three ml of this reagent was added to 2ml of sample. The mixture was incubated for 60 min at 35°C. The O.D. of the colour developed was read at 420nm. The amount of glucose released was read from glucose standards with concentrations ranging from 0-50mg/l.

(iv) Invertase activity

The invertase activity in the grains was also determined by using the glucose-oxidase-peroxidase method. The procedure was the same as that for alpha-glucosidase except that the substrate was 1% sucrose.

The total amylase, α-amylase, α-glucosidase and invertase activities of the isolates were determined as those of the grain enzymes.

(r) Utilization of sorghum processing waste for amylase production.

(i) Proximate composition of the sorghum processing waste

Sorghum waste which has been dried in the oven at 80°C for 48 hr was obtained from the Federal Institute of Industrial Research, Oshodi (FIIRO). The proximate composition, determined at the Analytical Laboratory of National Cereals Research Institute, Moore Plantation, Ibadan was carbohydrate 26.65%, protein 8.18%, ether extract 17.5% and total ash 1.35%.

(ii) Assessment of starch-hydrolysing abilities of microbial isolates

The micro-organisms used were those previously isolated from fermenting cassava. They are: Bacillus subtilis (1), B. subtilis (2), B. subtilis (3) and Corynebacterium manihot (FIIRO Strain). (Plates 2 and 3). Colony of B. subtilis (1) is dry surfaced while B. subtilis (2) and (3) are slightly moist.

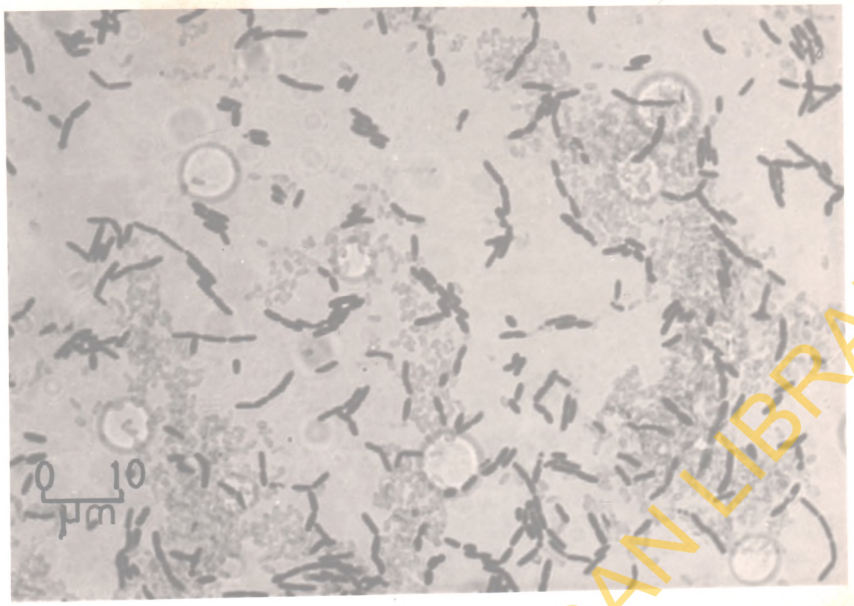
A loopful of 24 hr-old Nutrient broth suspension of each isolate was spotted on starch agar plate (containing 0.2% soluble starch). After incubating for 48 hr at 30°C, the plates were flooded with Grams iodine; after 3 minutes, the diameter of cleared zone around the bacterial colony was measured.

Isolates showing wide cleared zones were retained for further work. They were maintained on nutrient agar slants.

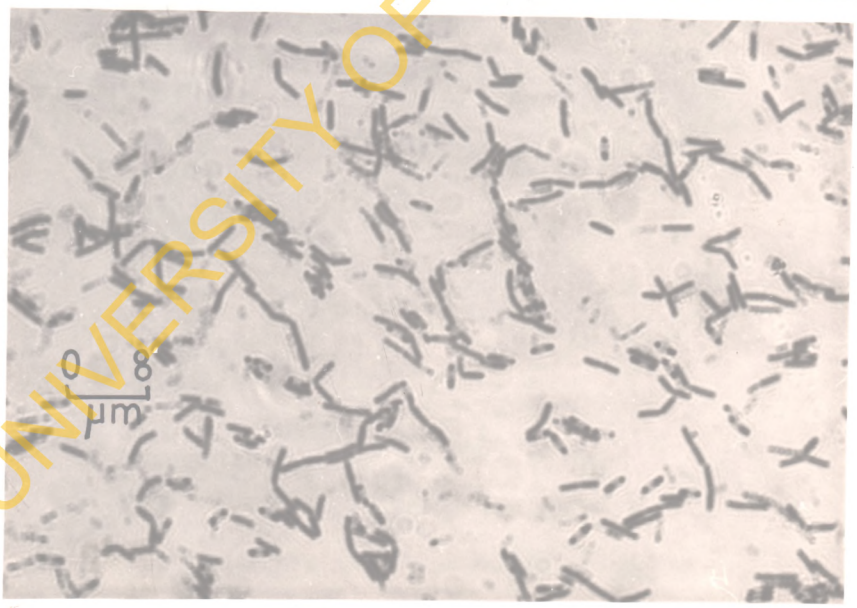
Five different media were tested for their ability to give good amylase production with sorghum waste as the only carbon source. The fermentation broth of Mahmoud et al. (1967) was modified for use. The composition of medium was as follows: sorghum waste 2%; bacteriological peptone, 1%, $K_2H\ PO_4.3H_2O$, 0.07%; KH_2PO_4, 0.03%; $MgSO_4.7H_2O$, 0.02%; $CaCl_2$, 0.02%;

A Plate 2. Bacillus subtilis (1) isolated from fermenting cassava.

B Plate 3. Bacillus subtilis (2) isolated from fermenting cassava.



A



B

Stock microelement solution (Forgarty et al., 1974) 0.1% ml, pH 6.5. For comparison, the same medium but with soluble starch substituted for sorghum waste was used alongside the above.

(iii) Time course of amylase activity

This is necessary to determine the peak period of amylase production. Flasks (150ml) each containing 100ml of the fermentation broth were inoculated with 1ml of 24 hr-growing culture and incubated at 30°C for up to 7 days. At 24 hr intervals, flasks were removed and the pH determined before filtration. The amylase activity was determined at 40°C. This was continued throughout the 7 days.

(iv) Effect of different pH values and buffer activity on amylase production

For the different pH values, citrate-phosphate buffer was used. This was made up by mixing appropriate volumes of 0.1M solution of citric acid and 0.2M solution of dibasic sodium phosphate to give pH ranges 3 - 8 (Hales, 1958). The various salts from the fermentation broth except the potassium salts were weighed into 100ml of the pH solution and then sterilized as above. Cultures were incubated at 30°C for 3 days.

The pH values and amylase activity of the medium at the end of incubation was measured using a Pye Unicam pH meter equipped with a glass electrode.

Different buffers at the optimum pH value were used for assessing the best buffer for amylase production; these were Tris buffer, phosphate buffer, citrate buffer and borate buffer (Lillie, 1948).

The salts of the fermentation broth were prepared in the different buffer solutions and sterilized as above. After allowing to cool, the flasks were inoculated and incubated at 30°C for 3 days. At the end of 3 days, the cultures were filtered and the pH of filtrate and amylase activity were determined as previously described.

(v) Effect of different temperatures on amylase:

The fermentation broth was prepared and inoculated. They were incubated at 25, 30, 35, 40, 50, 60, 70 and 80°C. At the end of 3 days, the flasks were removed and the pH and amylase activity were determined.

(vi) Effect of different nitrogen sources on amylase production

The fermentation broth used is the same as previously described except that the 1% peptone was substituted with 0.25% of the following:

$(NH_4)_2SO_4$, urea, and peptone. The control was without any nitrogen source added. After sterilization and inoculation, the flasks were incubated at 30°C optimal pH and temperature for 3 days. The amylase activity was then determined.

(vii) Effect of different concentrations of urea as the sole nitrogen source

Different concentrations of urea were added to the fermentation broth. After sterilization, the flasks were incubated. At the end of 3 days, the amylase activity in each duplicate flask was determined.

The percentage nitrogen in each urea concentration is as follows:

0.2% (0.09%N); 0.4% (0.18N); 1.0% (0.47%N); 2.0% (0.93%N); 3.0% (1.40%N). The values were worked out following the method of Taha et al., 1967.

(s) Starter culture development

(i) Preparation of sorghum flour for starter culture

The sorghum grains were milled dry in a commercial mill. The milled grains were sterilized using ethylene oxide vapour. The use of ethylene oxide vapour permits the sterilization of the sorghum powder without cooking the meal. Fifty grams of the milled flour

were weighed into 200ml crystallizing dishes. These dishes were placed inside a desiccator. A sealed flask containing liquefied ethylene oxide, which was previously cooled in an ice bath to 3 - 5^{o}C, was broken and 5ml of ethylene oxide was withdrawn into a crucible. The crucible containing the ethylene oxide and the dish containing the sorghum powder were placed in a desiccator. The desiccator was placed in a fume chamber. The sterilization time was 48 hr after which the desiccator was opened and the fume extractor switched on for a further 48 hr to evacuate the remaining ethylene oxide (Borch, 1955).

The sterilized flour was transferred into a presterilized 250ml flask, covered and stored in the deep freezer. The sterility of the sorghum flour was checked by plating out a portion on Plate Count agar (oxoid) for detecting any bacteria colonies and on Potato dextrose agar plates for moulds and yeasts; the plates were incubated at 30^{o}C for 48 hr for bacteria and 72 hr for fungi.

(ii) Preparation of inoculants

The pure microorganisms singly and indifferent combinations as listed in Table 2 below. The stock

TABLE 2. List of microorganisms used for inoculation

A	-	*Streptococcus lactis*
B	-	*Lactobacillus plantarum*
C	-	*Debaryomyces hansenii*
D	-	*Candida krusei*
A + B	-	*S. lactis* + *L. plantarum*
A + C	-	*S. lactis* + *D. hansenii*
A + D	-	*S. lactis* + *C. krusei*
B + C	-	*L. plantarum* + *D. hansenii*
B + D	-	*L. plantarum* + *C. krusei*
A + B + C	-	*S. lactis* + *L. plantarum* + *D. hansenii*
A + B + D	-	*S. lactis* + *L. plantarum* + *C. kruseii*

cultures were grown on Plate Count agar slants for 18 hr prior to inoculation. Then 10ml of sterile distilled water were added to the cultures in the MacCartney bottles and shaken vigorously.

(iii) Growth and acid production

Sterile distilled water (150ml) was added to the flask containing the sterile sorghum powder to give a 1:3 w/v dilution (sorghum: water). Then 3ml of slant-washed culture of the microorganisms listed in Table 2 were inoculated. An uninoculated ogi-baba served as control.

The extent of fermentation was measured by the following parameters: pH, total titratable acidity (as % lactic acid) and total plate count. These have been found in preliminary studies to increase with fermentation of ogi-baba and infact of other acidic gruels (Mbugua, 1981). The experiments were carried out in duplicate fermentations and three determinations were made at each interval. Values reported are the means of triplicate readings.

CHAPTER 3

RESULTS

(a) Microbiological analysis during preparation of ogi-baba

Table 3 shows the variation in the number of micro-organisms associated with the length of steeping while Table 4 shows the microbial count during souring. The results show that the total viable bacteria both Lactobacillus species and total lactic acid bacteria increased gradually until 48 hr. The moulds decreased sharply by 24 hr. The yeasts were isolated from 48 hr (see Table 3).

On the whole, various bacterial species were isolated from the steeping water. Fungi appeared only on the unfermented grains.

At souring, different bacterial species and another yeast were isolated.

Table 5 and 6 show the changes in pH and acidity during steeping and souring. The titratable acidity (as % lactic acid) increased throughout the steeping and souring, thereby resulting in the gradual fall in pH from 6.4 to 3.8 and increase in % lactic acid from 0.30 to 0.78. Most of the isolates tested were Gram positive and catalase negative. All the organisms tested fermented glucose. Most of the organisms isolated from the unfermented grains (Table 7) were homofermentative. Only organisms designated S4 and S6 were homofermentative amongst those isolated

Table 3. Microbiological changes during the steeping stage of sorghum fermentation.

a. Total count (per ml) incubated under aerobic condition

Time (Hrs)	Total Lactobacillus[a]	Total lactics[b]	Total yeasts[c]	Total count on PCA[d]	Total moulds
0	6.2×10^4	1.0×10^5	-	8.9×10^6	2.0×10^2
24	2.5×10^5	1.2×10^6	-	2.0×10^8	-
48	1.5×10^6	4.0×10^8	9.3×10^2	1.5×10^9	-
72	5.0×10^7	4.4×10^9	7.5×10^4	2.4×10^9	-

b. Total anaerobic count (per ml)

Time (Hrs)	Total Lactobacillus[a]	Total lactics[b]	Total count on PCA
0	7.1×10^4	1.8×10^5	9.2×10^5
24	1.85×10^6	2.3×10^6	2.3×10^6
48	1.15×10^7	0.9×10^7	1.2×10^7
72	4.7×10^7	1.2×10^7	1.1×10^7

Data represent means of three replicate determinations.

(a) Out of 15 isolated Lactobacillus, 13 were identified as L. plantarum
(b) Out of 22 isolated lactics, 9 were L. plantarum
6 were Streptococcus lactics, 4 were Pediococcus sp.
(c) Out of 12 yeasts isolated, 10 were Debaryomyces hansenii
(d) Out of 31 isolates on PCA, 8 were L. plantarum, 15 were Bacillus subtilis,
5 were S. lactics, 5 were Pediococcus sp. and 5 Enterobacteriaceae

Key: PCA = Plate count agar (oxoid formulation).

Table 4. Microbiological changes during souring stage of ogi-baba fermentation.

a. Aerobic plate count (per ml)

Time (Hrs)	Total Lactobacilii[a]	Total lactics[b]	Total yeasts[c]	Total Count on PCA[d]	Total moulds
0	4.2×10^8	1.76×10^7	2.0×10^6	1.3×10^9	-
12	4.5×10^8	1.95×10^8	3.1×10^6	1.7×10^9	-
24	6.3×10^8	2.3×10^9	5.4×10^6	1.9×10^9	-
36	7.2×10^8	2.5×10^9	9.7×10^6	2.0×10^9	-
48	8.0×10^8	2.7×10^9	1.2×10^6	2.4×10^9	-

b. Anaerobic plate count (per ml)

Time (Hrs)	Total Lactobacillii	Total lactics	Total count on PCA
0	7.1×10^6	1.0×10^7	1.2×10^6
12	8.9×10^6	1.3×10^7	1.3×10^6
24	1.1×10^7	4.7×10^7	1.7×10^6
36	1.3×10^7	7.9×10^7	1.9×10^6
48	2.1×10^7	2.7×10^8	

Data represent means of three replicate determinations.

(a) Out of 12 isolated Lactobacilii, 10 were L. plantarum.

(b) Out of 17 lactics isolated 8 were S. lactis, 2 were L. plantarum, 3 were other Lactobacillus spp., 1 was Leuconostoc sp.

(c) Of the 21 yeasts isolated 18 were Candida krusei

(d) Of the 28 isolates on PCA 5 were identified as B. subtilis, 7 as S. lactis, 1 as L. plantarum and 5 Enterobacteriaceae.

TABLE 5

Changes in pH and acidity during steeping

Time (hr)	pH	Titratable acidity (% Lactic acid)	Oxygen level (% Saturation)
0	6.4	0.30 ± 0.04	28.0
24	6.0	0.45 ± 0.03	12.0
48	5.6	0.51 ± 0.05	2.1
72	5.0	0.53 ± 0.06	2.0
84	4.0	0.55 ± 0.03	2.0

Each figure is a mean of 3 replicate determinations.

TABLE 6.

Changes in pH and acidity during souring

Time (hr)	pH	Titratable acidity (% Lactic acid)
0	5.0	0.54 ± 0.07
24	4.8	0.62 ± 0.04
48	3.8	0.78 ± 0.05

Each figure is a mean of 3 replicate determinations.

TABLE 7

Results of biochemical and physiological tests on microorganisms isolated from unfermented grains

ISOLATES	Morphology	Motility	Spore stain	Gram stain	Catalase	Het-Homo	Oxi-ferm	Oxi-Rel	Starch hydrolysis	Casein	Growth at 45°C	Growth at 15°C	Nitrate test	Gas from Arginine	Na hippurate	Sugar ferm	Glucose	Raffinose	Manittol	Mannose	Sucrose	Fructose	MR	VP	IDENTIFIED MICRO-ORGANISMS
SG.1	R	-		+	-	HOMO	F	Anae	-	+	+	+	-	+	+		+	+	-	+	+	+	+	-	Lactobacillus plantarum
SG.2	R	-	+	+	-		O	Fac anae	+	-	+	+	-		-		+	+	-	-	+	+	+	-	Bacillus subtilis
SG.3	C	-		+	-	HOMO	F	Fac anae	-	+	-	+	-	+	+		+	+	+	+	-	+	+	-	Streptococcus lactis

SP - *Penicillium* sp.

SB - *Aspergillus* sp.

Key

R - Rods
C - Cocci
F - Fermentative
O - Oxidative
\- : Negative
\+ : Positive
Fac. anae - Facultative anaerobe
Anae - Anaerobic

TABLE 8

Result of biochemical and physiological tests on microorganisms isolated during steeping and souring

ISOLATES	Morphology	Motility	Spore Stain	Gram Stain	Catalase	Hete-homo	Oxy-ferm	Starch hydrolysis	Casein	Indole	Growth at 45°C	Growth at 15°C	Nitrate	Gas from arginine	Na hyppurate	Sugar ferm	Glucose	Raffinose	Mannitol	Mannose	Fructose	MR	VP	IDENTIFIED MICRO-ORGANISMS
S_1	R	-	-	+	-	Het	F	-	-	-	+	+	-	-	+		+	+	-	+	-	+	-	Lactobacillus plantarum
S_2	R	-	-	+	-	het	F	-	-	-	-	+	-	-	-		+	+G	+G	+	-	+	-	L. plantarum
S_2B	R	-	+	+	-		F	+	+	-	+	+	-		-		+	-	-	-	+	+	-	B. subtilis
S_3	C in Chains	-		+	-	het	F	-	-	+	+	+	-	+	+		+	-	-	-	-	+	-	Streptococcus lactis
S_4	C diplo	-		+	+	homo	O	+	+	-	-	+	-	-	-		+	+G	+	-	+	+	-	Pediococcus
S_5	C	-		+	-	het	F	-	-	-	-	+	-	-	+		+	-	+	-	+	-	-	Leuconostoc
								Souring																
S_6	C	-		+	-	homo	F	-	-	-	-	+	-	+	+		+	+	+	-	-	+	-	S. lactis
S_7	R	-	-	+	-	hete	F	-	-	-	-	+	-	-	+		+	+	+	-	+	-	-	Lactobacillu
S_8	C	-		+	+	hete	Oxi	+	-	-	-	+	-	-	+		+	-	+	-	+	+	-	Leuconostoc

during steeping and souring; all others were heterofermentative (Table 8). From the saccharolytic test, only isolates designated SG2, SG2B and the two yeasts hydrolysed starch.

The results of biochemical tests were used to identify the microorganisms using the keys provided in Bergeys manual (Buchanan and Gibbons, 1974) and identification methods for microbiologists (Sharpe, 1979).

The biochemical characteristics of the representative isolates of the yeasts are shown in Table 9. Candida krusei is cream coloured with well developed pseudomycelium but with no spores. It is capable of fermenting and assimilating glucose. D. hansenii is pinkish cream with no mycelium and possesses ascospores. It is capable of assimilating many carbon sources. It differs from the type species in its ability to assimilate galacitol but not soluble starch.

Plates 4 - 10 show the different bacterial and yeast isolates.

The moulds isolated were as follows:-

Aspergillus sp. The colonies on PDA has black colour. On examination, the conidiophores were erect, single and measure 3.1 - 7.9 µm in diameter. The conidiophore terminated with a globose vesicle of 11 - 21 µm in diameter. It has 2 biseriate sterigmata on convex shaped upper surface. The conidia are

TABLE 9. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE YEASTS OF FERMENTING GUINEA CORN

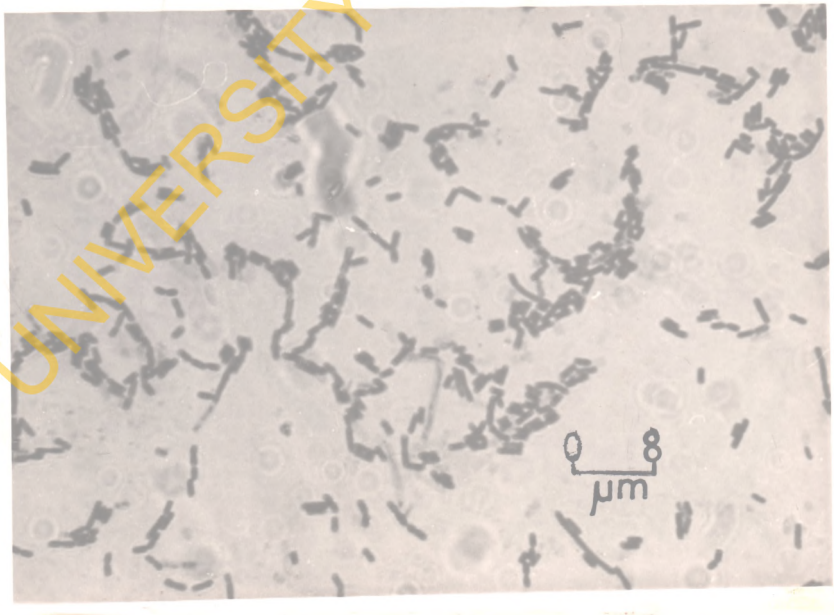
Laboratory reference No.	S015	S020
Colony morphology	Cream, dull, rough on 48 h agar plate	Pinkish cream, shiny, smooth on 48h agar plate
Cell shape and size	Long to elongated single and in chains (3 - 6) x (6 - 14)µm	Round to oval, single but mainly in groups (2 - 5) x (2 - 7)µm
Pseudomycedium	Well-developed on PDA with irregular branches and oval blastospores at verticils	
Sporulation	-	Round, smooth, ascospores, 1 per ascus
Fermentation		
Glucose	+	-
Galactose	-	-
Maltose	-	-
Sucrose	-	-
Lactose	-	-
Mellibiose	-	-
Raffinose'	-	-
Assimilation		
Glucose	+	+
Galactose	-	+
Maltose	-	+
Sucrose	-	+
Lactose	-	+
Mellibiose	-	+
Raffinose	-	+
Soluble starch	-	-*
Galactitol	-	+*
Oxygen relationship :	Fac. anaerobe	Fac. anaerobe
Yeasts identified :	Candida krusei	Debaryomyces hansenii

*Differs from standard description.

A. Plate 4. Photomicrograph of isolate S2B identified as Bacillus subtilis from the steeping stage.

B. Plate 5. Photomicrograph of isolate S1 identified as Lactobacillus plantarum

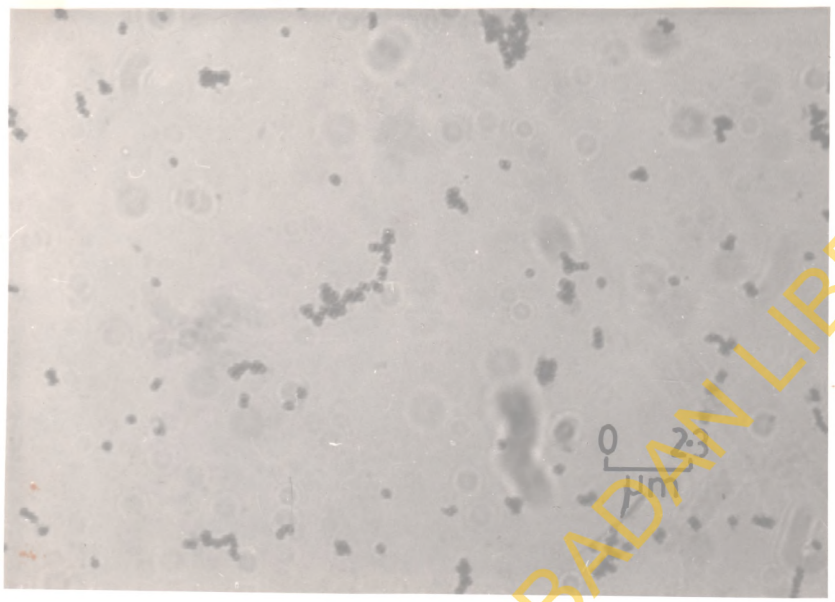
A



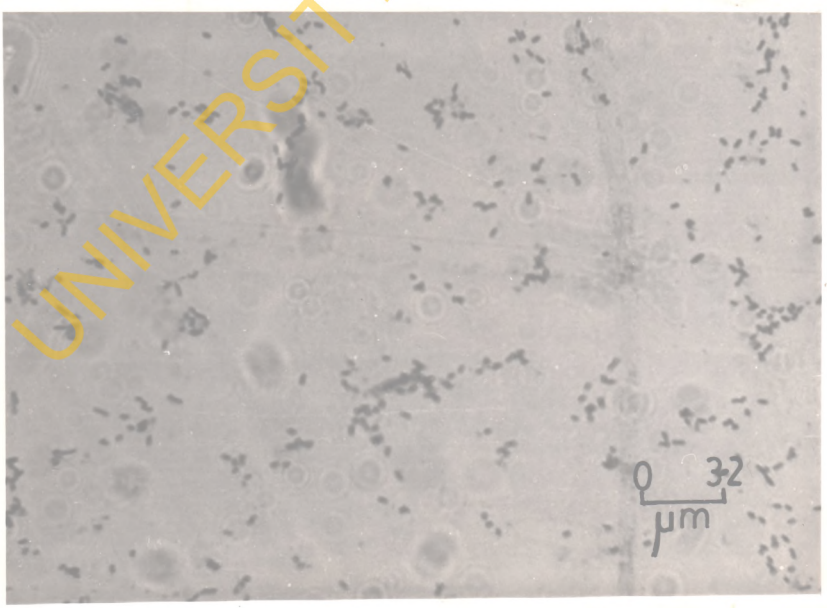
B

A. Plate 6. Photimicrograph of isolate S6 identified as Streptococcus lactis.

B. Plate 7. Photomicrograph of isolate S4 A Pediococcus sp.

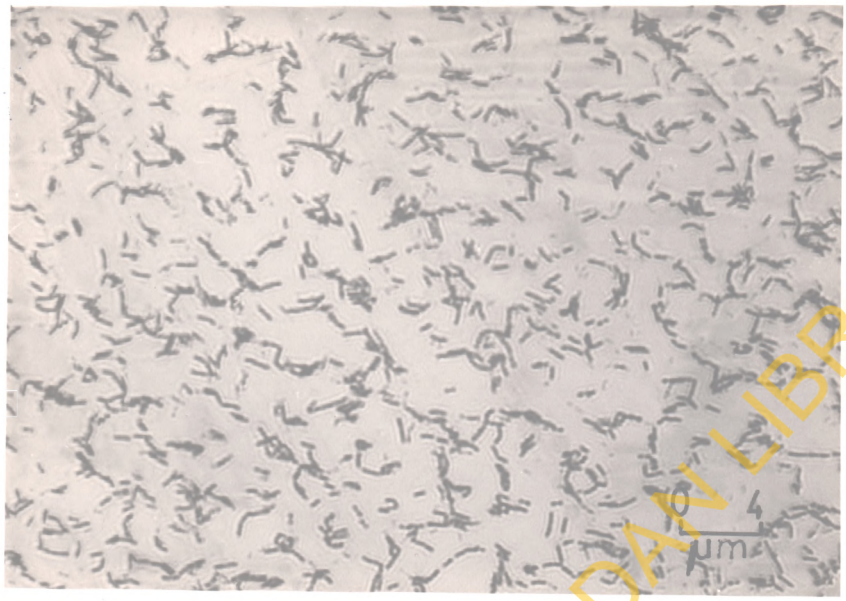


A



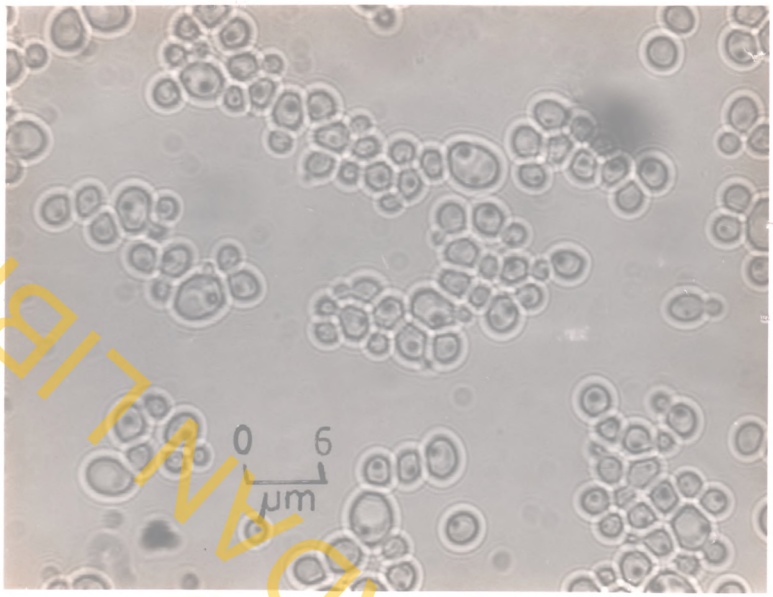
B

Plate 8. Photomicrograph of isolate S2 *Lactobacillus* species.

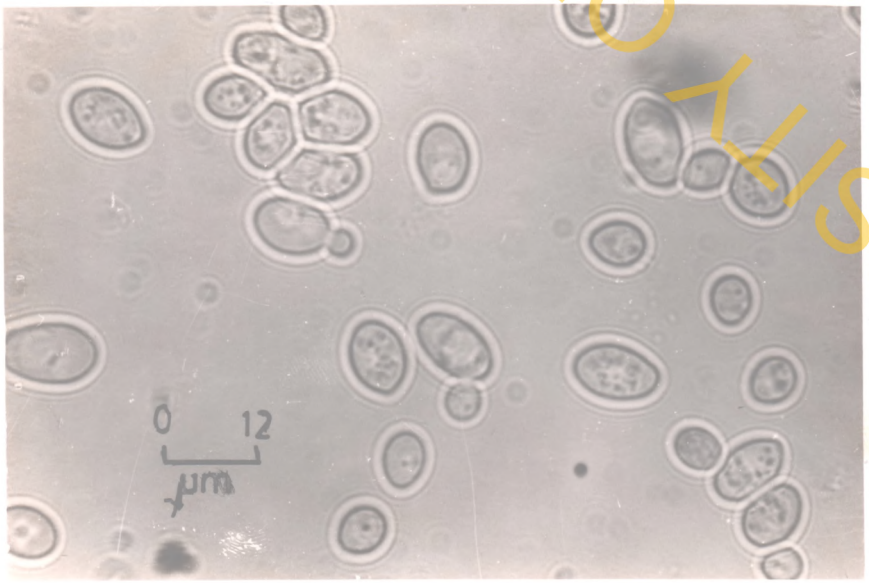


A. Plate 9. Yeast isolated during the steeping period of ogi-baba - Debaryomyces hansenii

B. Plate 10. Yeast isolated during the souring period of ogi-baba - Candida krusei



A



B

slightly elliptical to globose of about 2.3 - 2.6 µm in diameter and smooth walled.

Penicillium sp. The colonies appeared greenish yellow on PDA plate. On examination, the conidiophores were erect measuring 100 - 150 um in length. The conidiophore terminated at the apex to form finger-like sterigmata measuring 10 - 19 µm in diameter. The conidia are smooth-walled measuring 2.7 - 2.9 µm in diameter.

The results show that the unfermented grains contained bacteria of the following genera : Bacillus, Lactobacillus and Streptococcus while the fungal species were Aspergillus and Penicillium. The fungal species disappeared by 24 hr.

During steeping, Lactobacillus plantarum, Bacillus subtilis and Steptococcus lactis appeared first, followed by a yeast Debaryomyces hansenii. At souring, the major organism was S. lactis and another yeast Candida krusei.

(b) Physiological studies on dominant isolates

Figures 2 - 6 show the effects of temperature on the growth of the pure isolates. From the graph (Fig. 2) L. plantarum had its optimal growth at 30°C, minimum growth at 60°C and an optimal growth range between 25 - 40°C. Streptococcus lactis has an optimum growth range of 25 - 40°C (Fig. 3) while the other Lactobacillus species had an optimal growth temperature range of

Fig. 2. Effect of temperature on the growth of Lactobacillus plantarum.

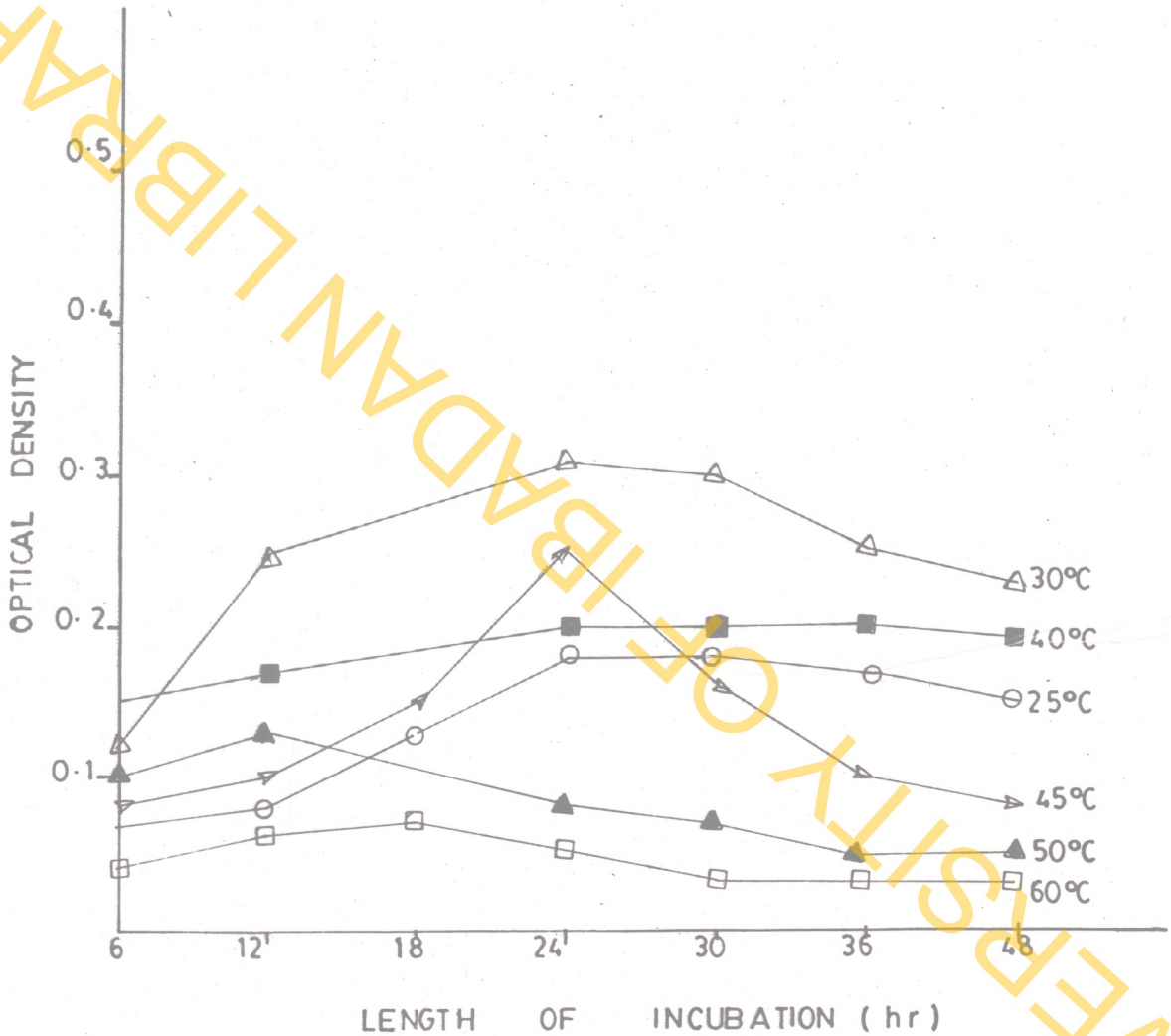
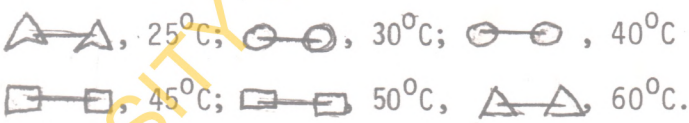


Fig. 3. Effect of temperature on the growth of Streptococcus lactis.



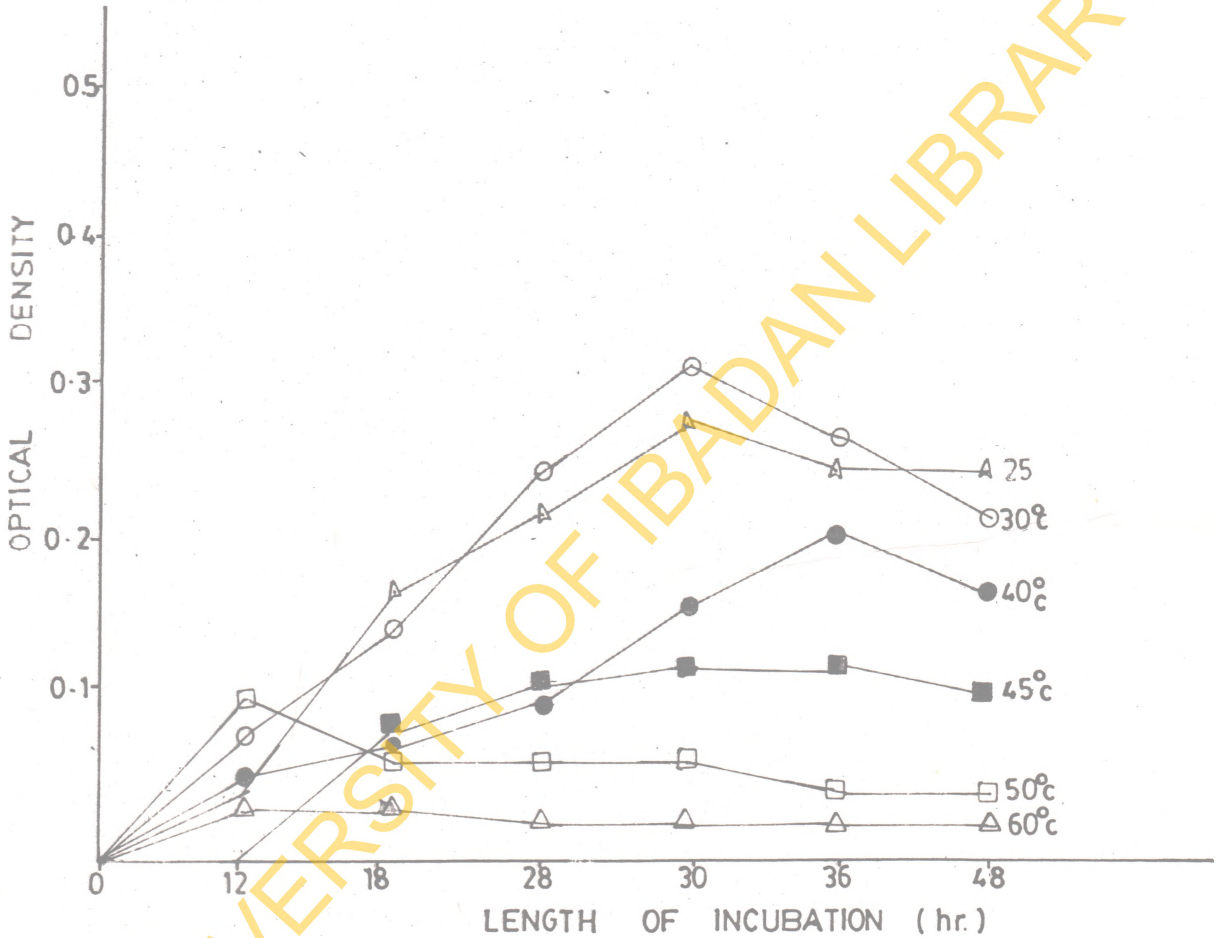


Fig. 4. Effect of different temperatures on the growth of Lactobacillus spp.

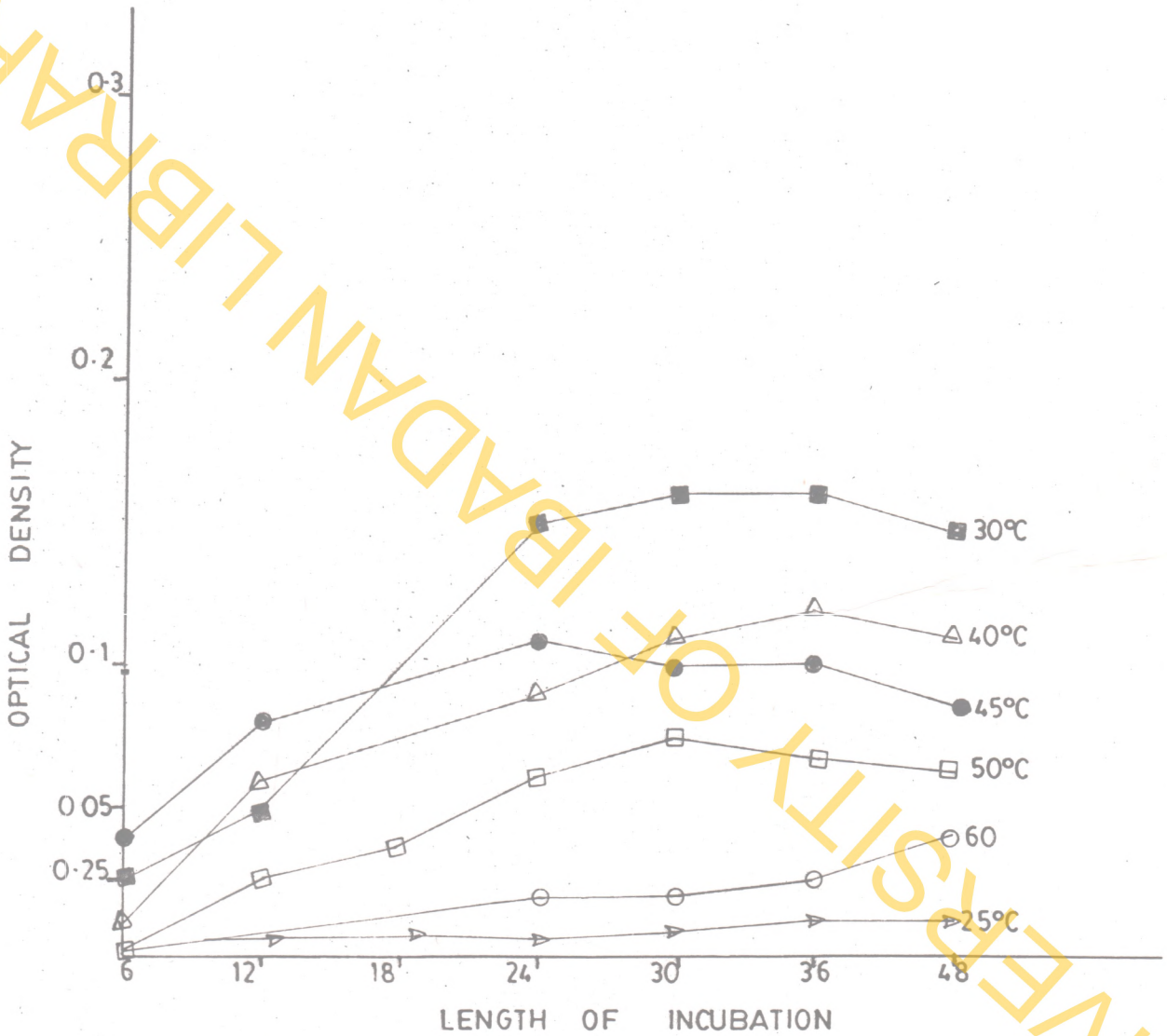


Fig. 5. Effect of different temperatures on the growth of Debaryomyces hansenii.

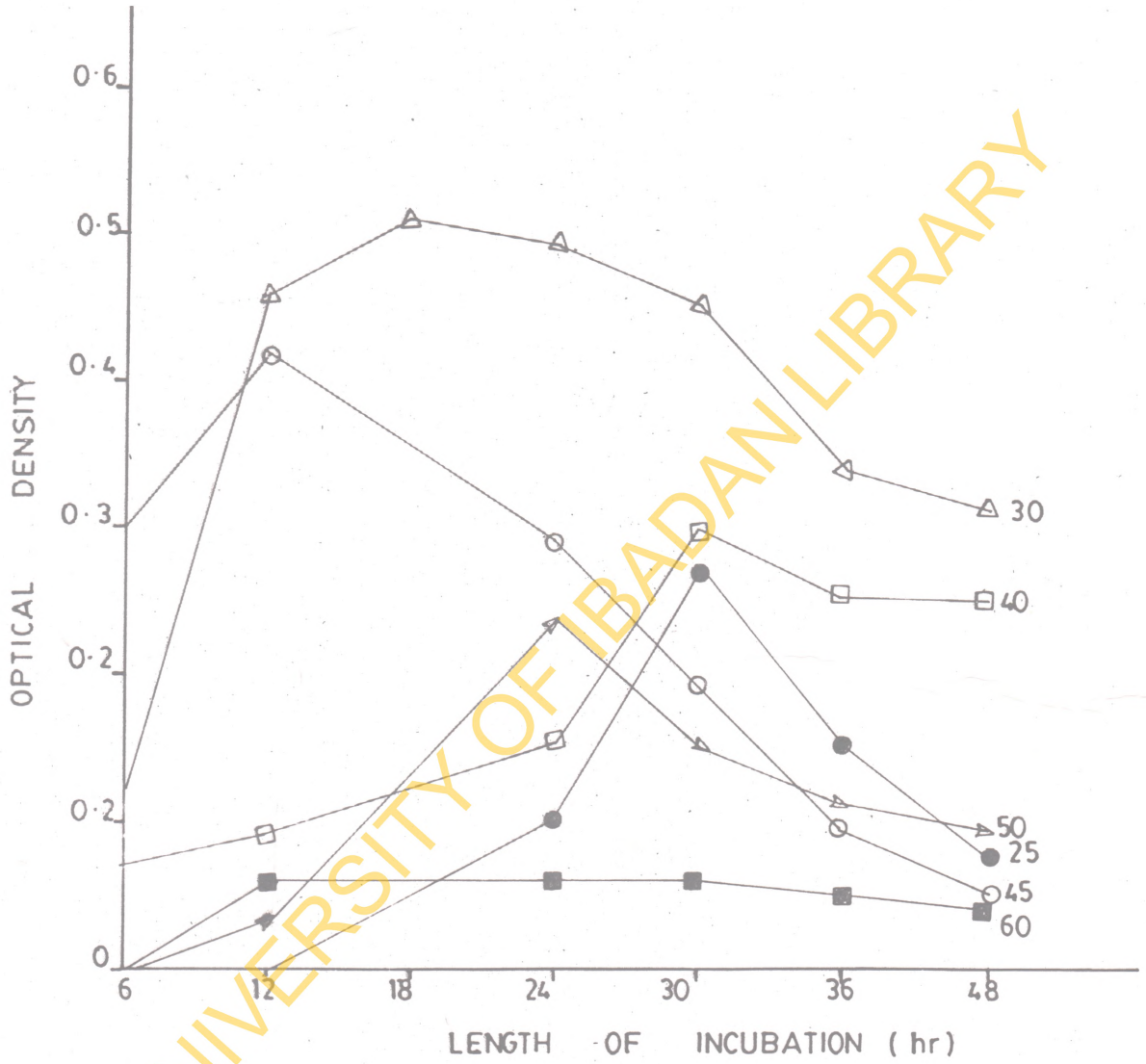
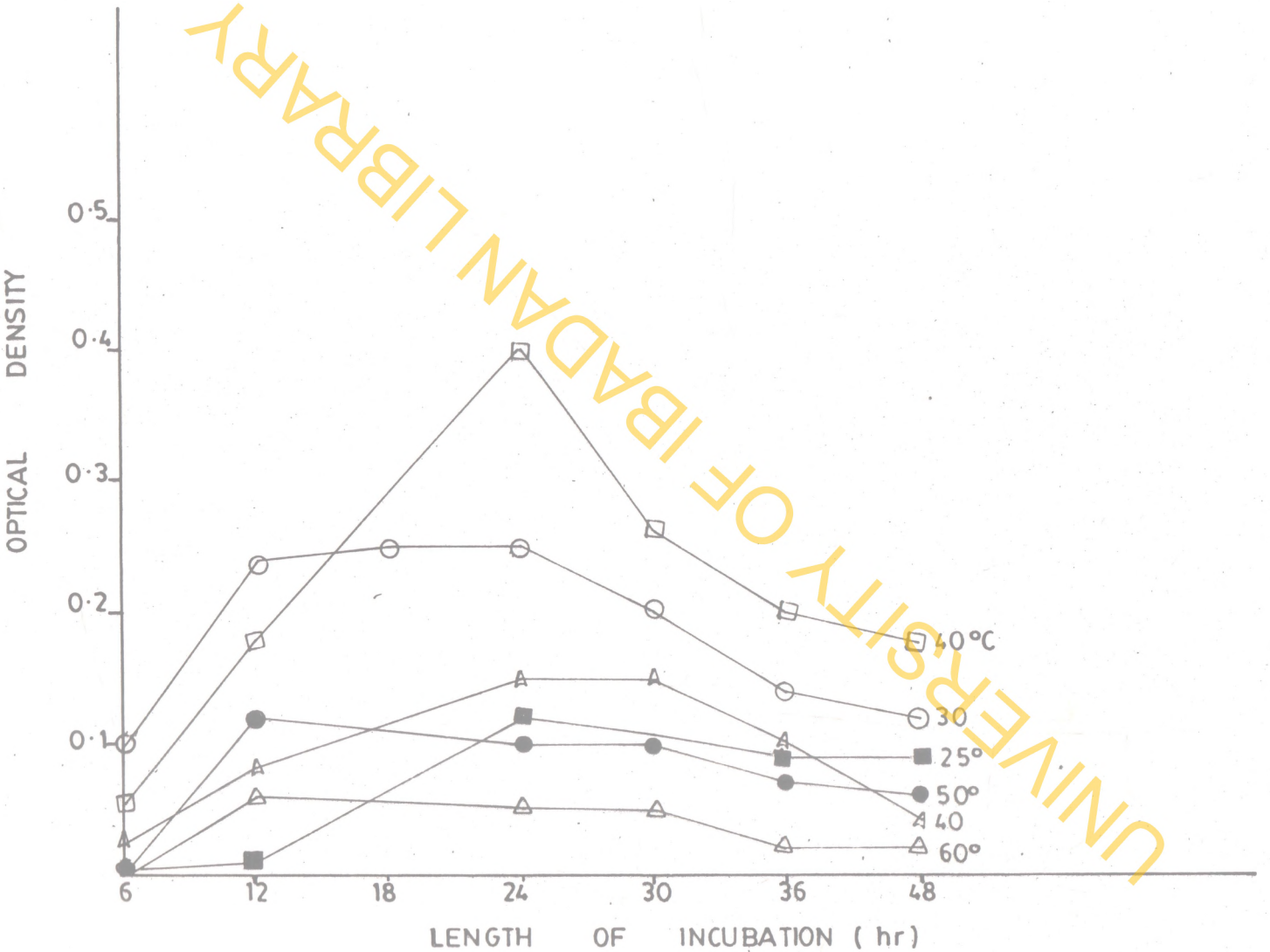


Fig. 6. Effect of different temperatures on the growth of Candida krusei.



30 - 45°C (Fig. 4).

Of the two yeasts, D. hansenii had rapid growth at 30°C and 45°C while C. krusei had rapid growth at 30 and 40°C (Fig. 5 and 6) but was most rapid at 40°C.

As shown in Fig. 7, L. plantarum and Lactobacillus sp. had an optimal pH growth range of 5 to 5.5. Streptococcus lactis had its optimum pH at 5. Debaryomyces hansenii and C. krusei grew best at pH 5 and 4 respectively (Fig. 8).

(c) Sugar changes and their utilization during fermentation

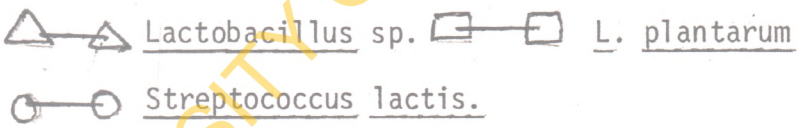
From the chromatogram (Plate 11), the unfermented grains contained raffinose, sucrose, glucose and fructose; maltose was present in trace amounts. Within 24 hr of fermentation, there was a sharp decrease in sucrose while fructose decreased conspicuously.

At souring, glucose was the predominant sugar present, others were maltose, sucrose and fructose (Plate 12).

At steeping, there was an initial decline in the total ethanol-soluble sugars (Fig. 9) and a subsequent increase. There was a progressive decline in the sugar content with length of souring. The starch content decreased during steeping (Fig. 10).

The oligosaccharides present in the unfermented grains were stachyose, raffinose, sucrose and fructose. The stachyose

Fig. 7. Effect of different pH on the growth of bacterial isolates.



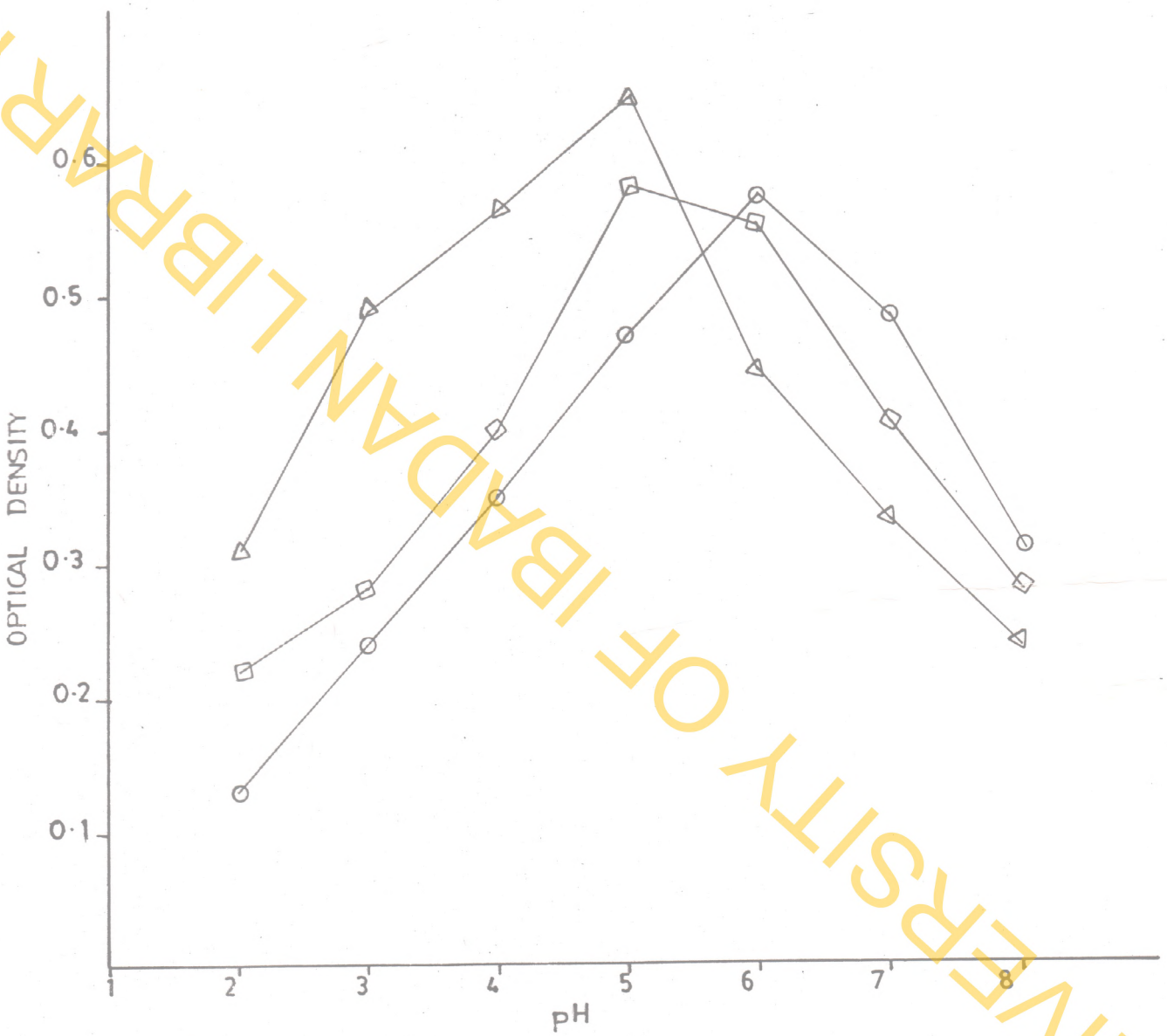


Fig. 8. Effect of different pH on the growth of yeast isolates.

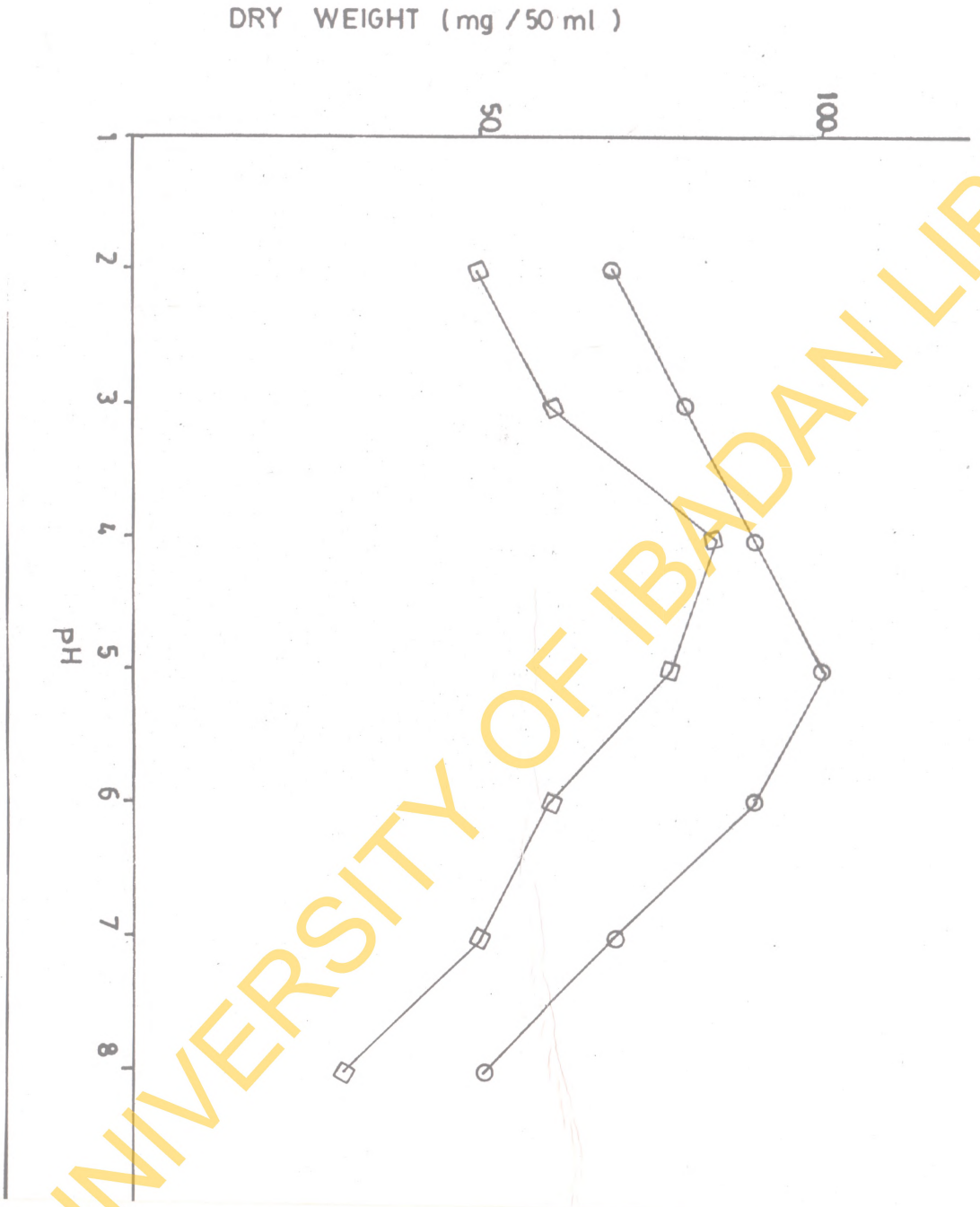


Plate 11. Chromatogram showing changes in the sugar content during steeping stage.

A. Raffinose
B. Maltose
C. Sucrose
D. Glucose
E. Fructose
F. Xylose.

Key for 1, 2, 3, 4, 5

1 - unfermented sorghum
2 - 24 hr steeping
3 - 48 " "
4 - 72 " "
5 - Reference standard sugars.

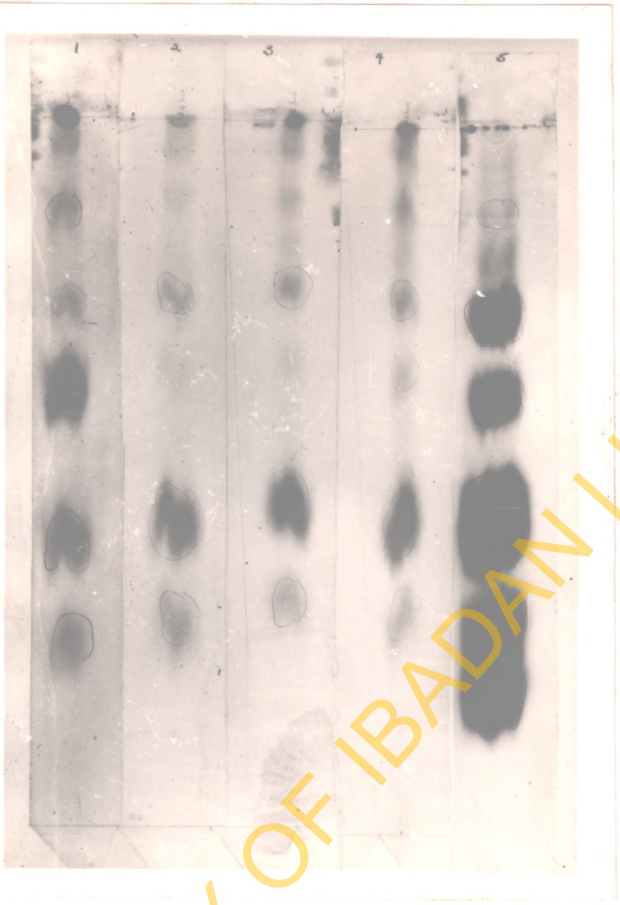


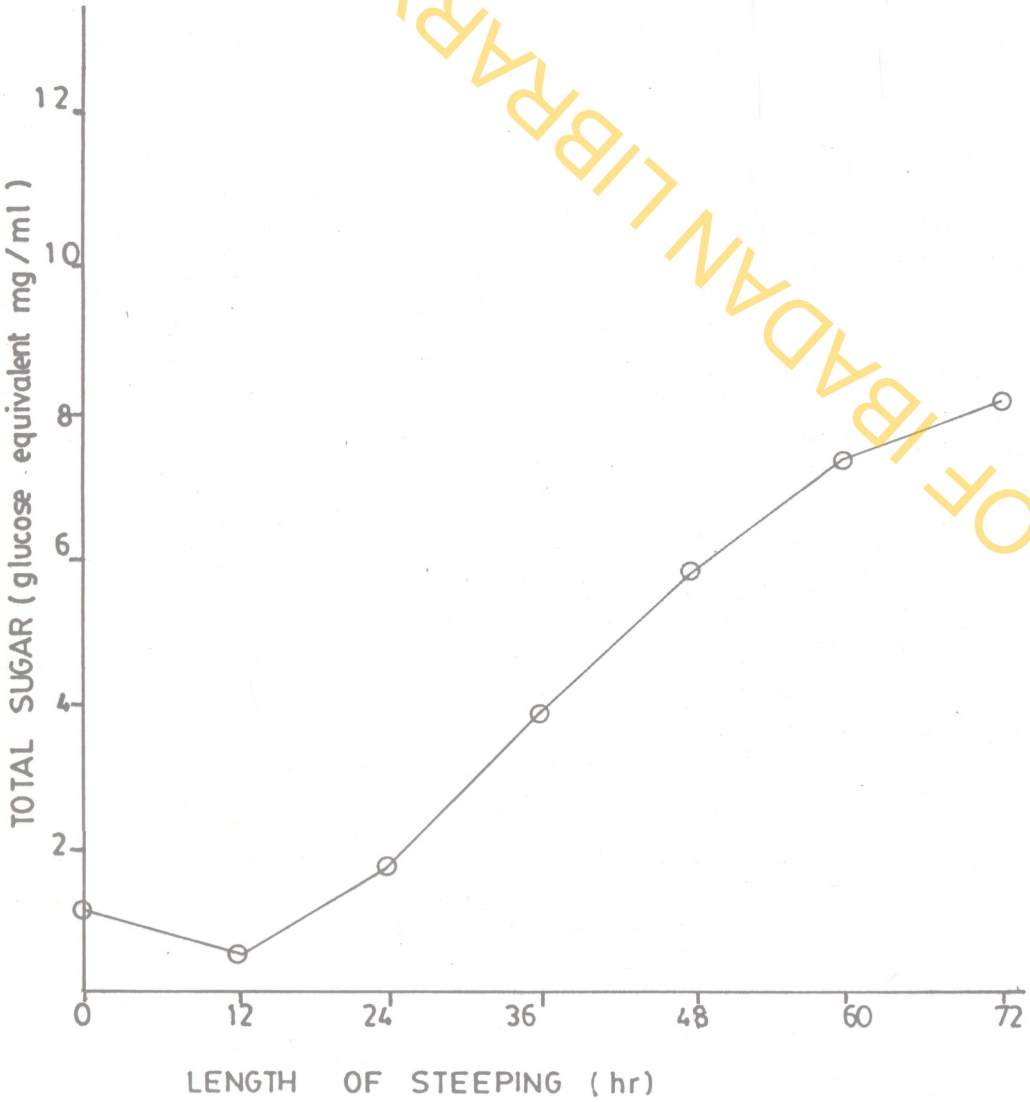
Plate 12. Chromatogram showing changes in the sugar content during souring stage

A. Raffinose
B. Maltose
C. Sucrose
D. Glucose
E. Fructose
F. Xylose

Key for 1, 2, 3, 4, 5

1 - 0 hr souring
2 - 24 hr "
3 - 48 " "
4 - 72 " "
5 - Reference standard sugars.

Fig. 9. Total ethanol soluble sugars during steeping and souring of sorghum fermentation.



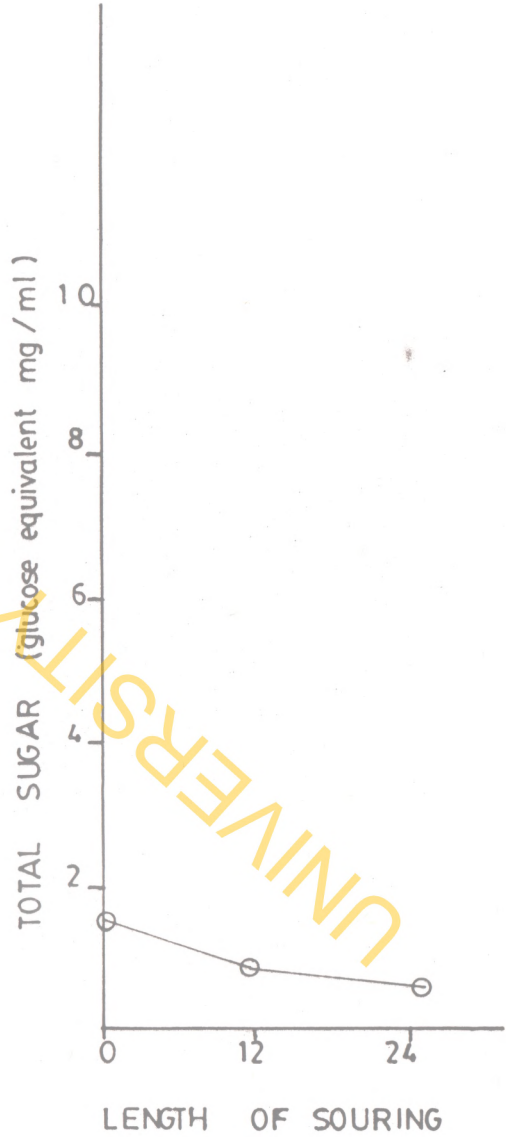
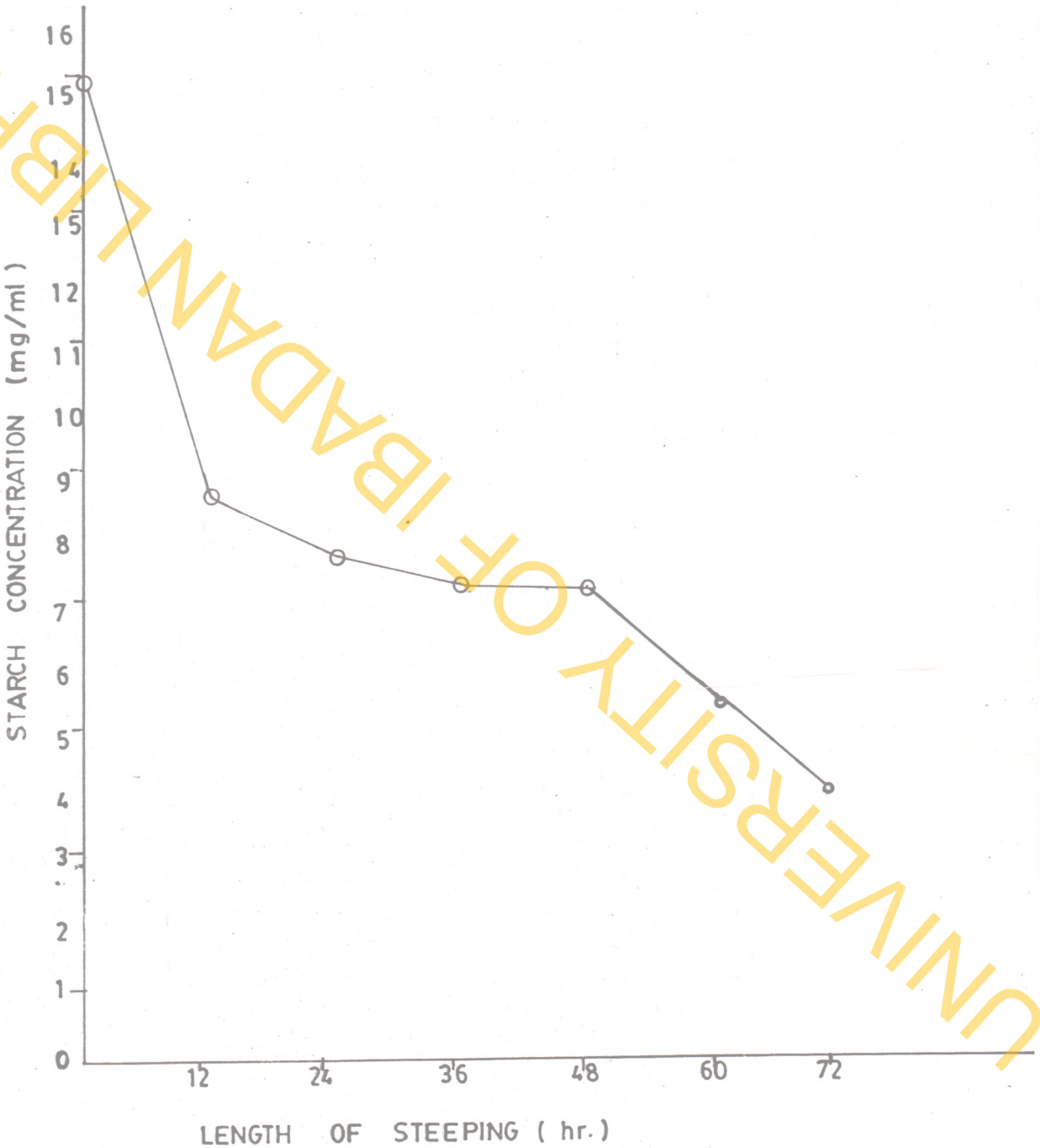


Fig. 10. Changes in the concentration of starch during fermentation of sorghum for ogi-baba production.



disappeared by 24 hr and fructose disappeared by 36 hr (Chromatogram not shown). At souring, only fructose was detectable on the T.L.C. plate (Chromatogram not shown).

The utilization of sugars as determined by titratable acidity of growth medium of the bacteria after 48 hr incubation, shows that glucose was preferentially utilised by Streptococcus lactis and not utilised by Debaryomyces hansenii; galactose was utilised as preferential sugar for growth by L. plantarum (Table 10). Debaryomyces hansenii and C. krusei (Table 11) utilised sucrose, xylose and galactose preferentially.

Titratable acidity showed that the growth of S. lactis was highest in glucose ($P < 0.05$) whereas the growth of all the other microorganisms were significantly higher ($P = 0.01$) in galactose and maltose.

A comparison of titratable acidity (Table 10) and pH changes after 48 hr (Figs. 11 - 14) best demonstrates the utilization of glucose, sucrose, fructose and maltose by the two bacterial species and yeasts studied. As shown in Fig. 11, S. lactis utilised glucose for growth more than sucrose and maltose. Glucose was also utilised for growth by L. plantarum and there was less significant growth in fructose medium (Fig. 12). Of the two yeasts, D. hansenii utilised maltose significantly better (Fig. 13 and Fig. 14). Growth of D. hansenii was significantly

TABLE 10.

Utilization of various sugars by dominant micro-organisms - by determining titratable acidity after 48 hr.

(ml 0.01N NaOH)

	Lactobacillus plantarum	Streptococcus lactis	Debaryomyces hansenii	Candida krusei
Xylose	3.3	4.0	2.5	2.5
Glucose	2.8	8.1	1.6	.05
Fructose	1.8	2.9	1.0	0.8
Galactose	3.4	2.7	2.0	2.0
Lactose	1.4	0.8	1.4	0.6
Maltose	2.5	4.4	2.1	1.6
Sucrose	1.2	2.7	3.0	2.0
Raffinose	0.9	1.1	0.8	1.5
Control	0.15	0.13	0.15	0.10

Table 11. Dry weight of yeasts

(g/50 ml)

	D. hansenii	C. krusei
Xylose	0.025	0.025
Glucose	0.021	0.016
Fructose	0.010	0.008
Galactose	0.020	0.020
Lactose	0.014	0.006
Maltose	0.021	0.016
Sucrose	0.030	0.020
Raffinose	0.008	0.015
Control	0.005	0.015

Each figure is a mean of 3 replicate determinations.

Fig. 11. Acid production in various sugar media by *S. lactis.*

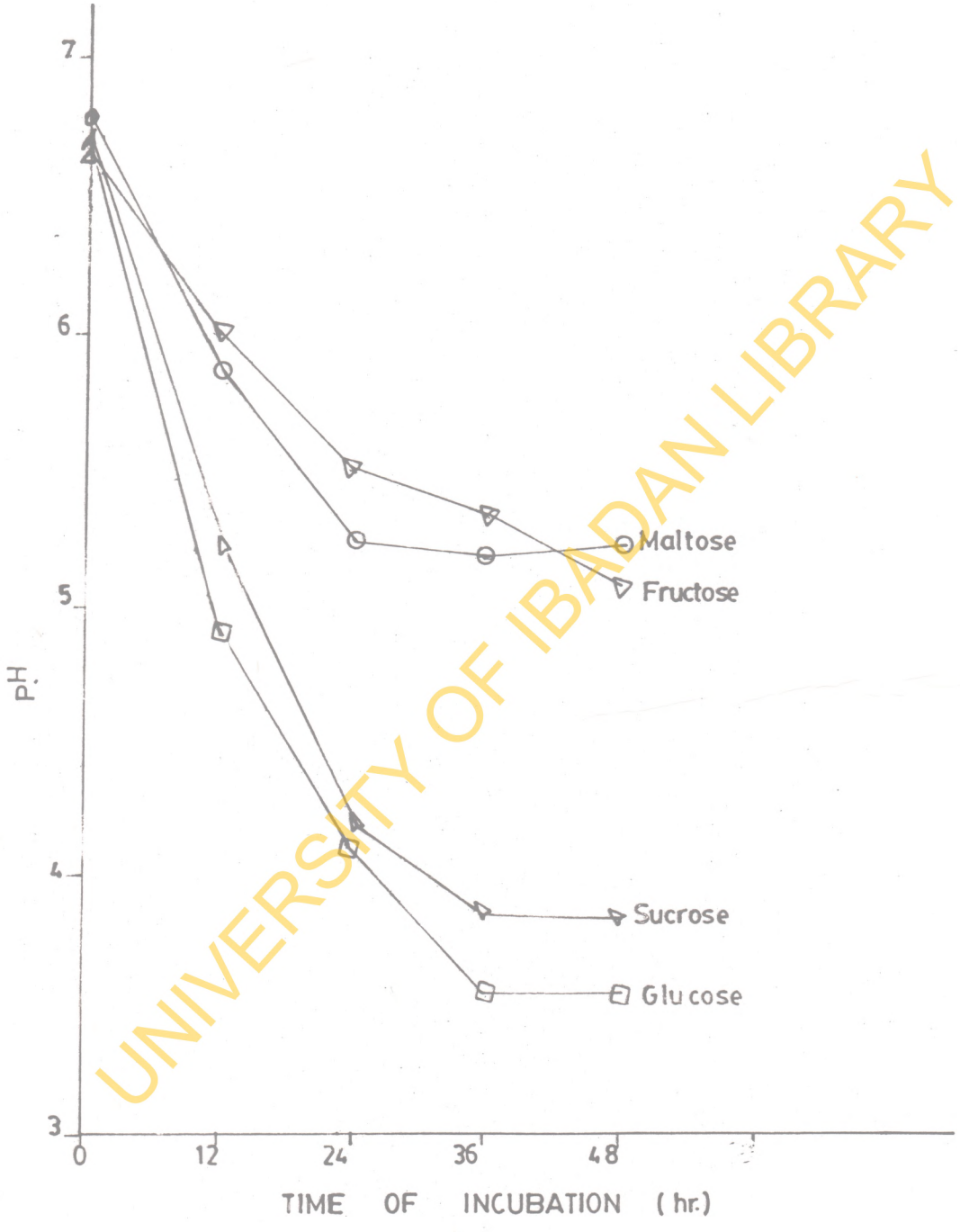


Fig. 13. Acid production in various sugar media by D. hansenii

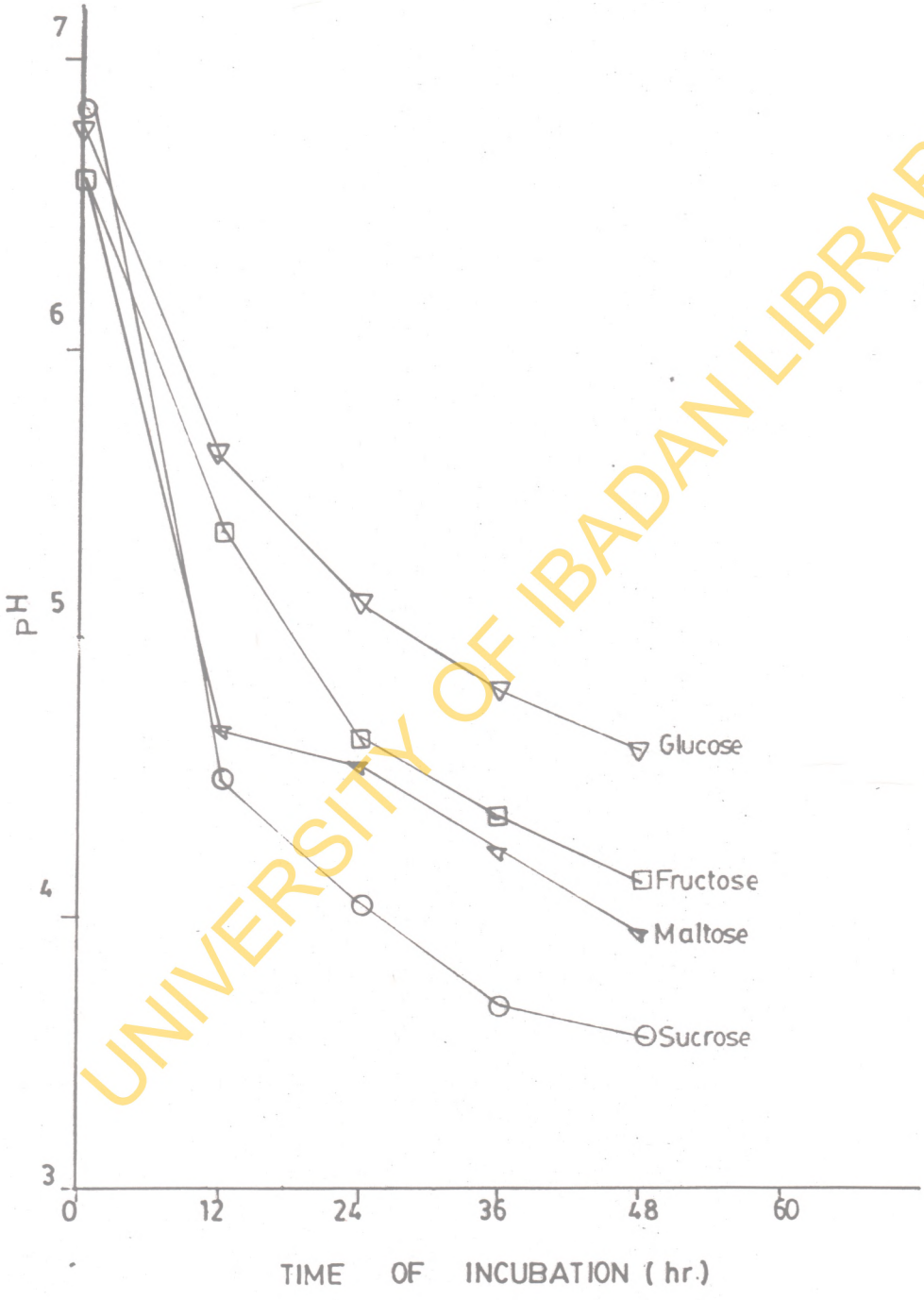
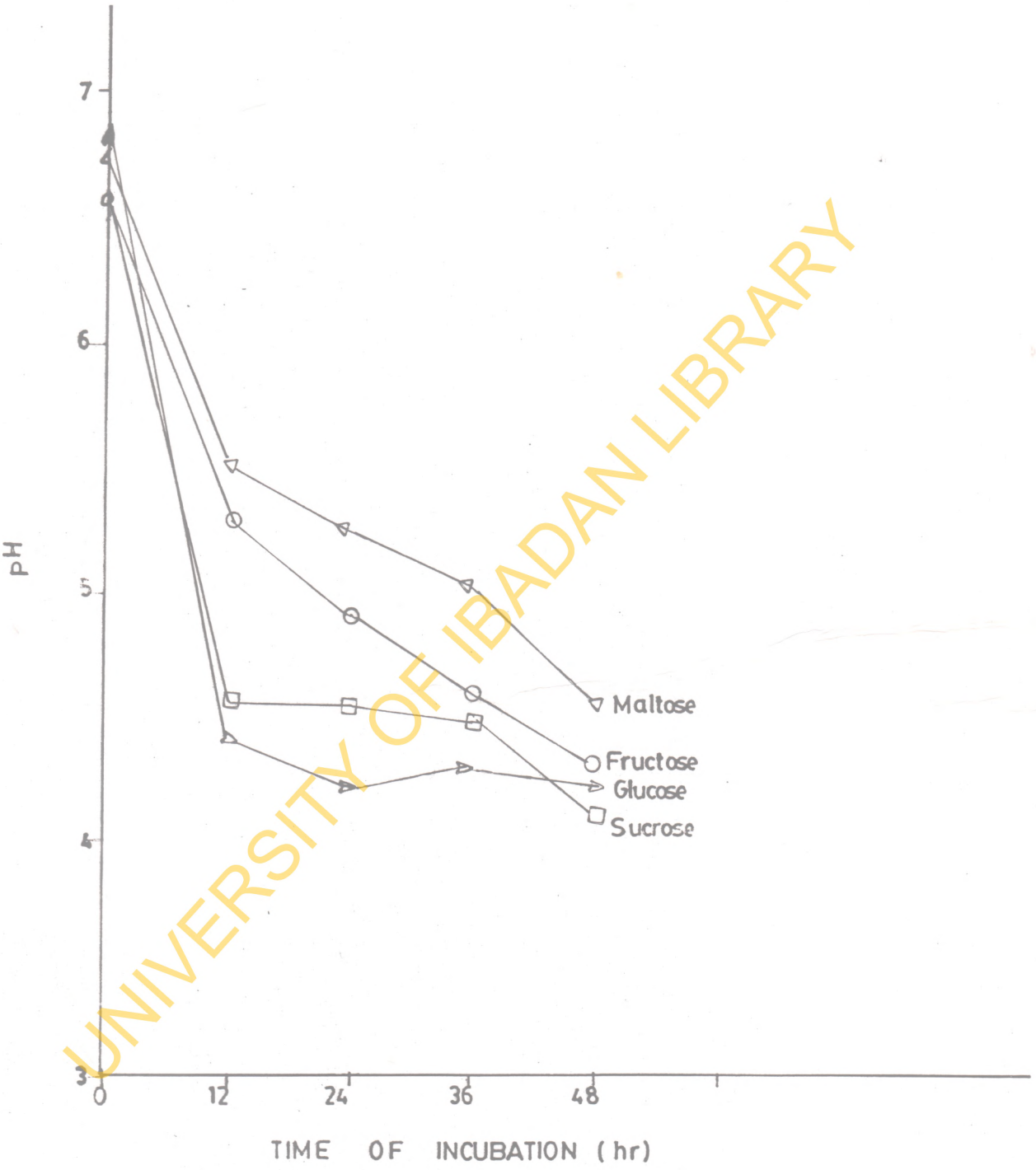


Fig. 14. Acid production in various sugar media by *C. krusei*



better in sucrose medium and least in glucose medium (Fig. 13).

(d) Enzymatic activities during sorghum fermentation for ogi-baba

Figure 15 show the ∝-amylase and total amylase activities respectively in the fermenting grains. There was no ∝-amylase within the first 12 hr and the amount subsequently remained stable with little fluctuations. The total amylase during the fermentation was at its peak at 24 hr. The ∝-glucosidase activity (Fig. 16) increased to a peak at 36 hr and showed a decrease at 48 hr after which it increased again.

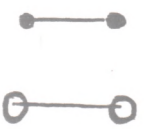
The amylase activities of the different isolates are shown in Fig. 18. The two yeasts produced the highest amylase activity and they were able to hydrolyse starch on agar plate culture. All the isolates produced ∝-glucosidase (Fig. 19) the highest activity of invertase were found in the two yeasts (Fig. 20).

(e) Amylase production from sorghum processing waste

The result in Table 12 shows that B. subtilis (1) and (2) have the maximum width of clearing on starch agar plate. Therefore for further work, B. subtilis (1) and (2) were used. The result of the sugar fermentation tests on the two Bacillus sp. is as shown in Table 12.

The optimal length of time for maximum amylase production by B. subtilis (1) was 72 hr on sorghum medium and 96 hr on soluble

Fig. 15. Alpha and total-amylase activity in fermenting grains during steeping.



Alpha amylase

Total amylase

Fig. 16. Alpha-glucosidase activity in fermenting grains during steeping.

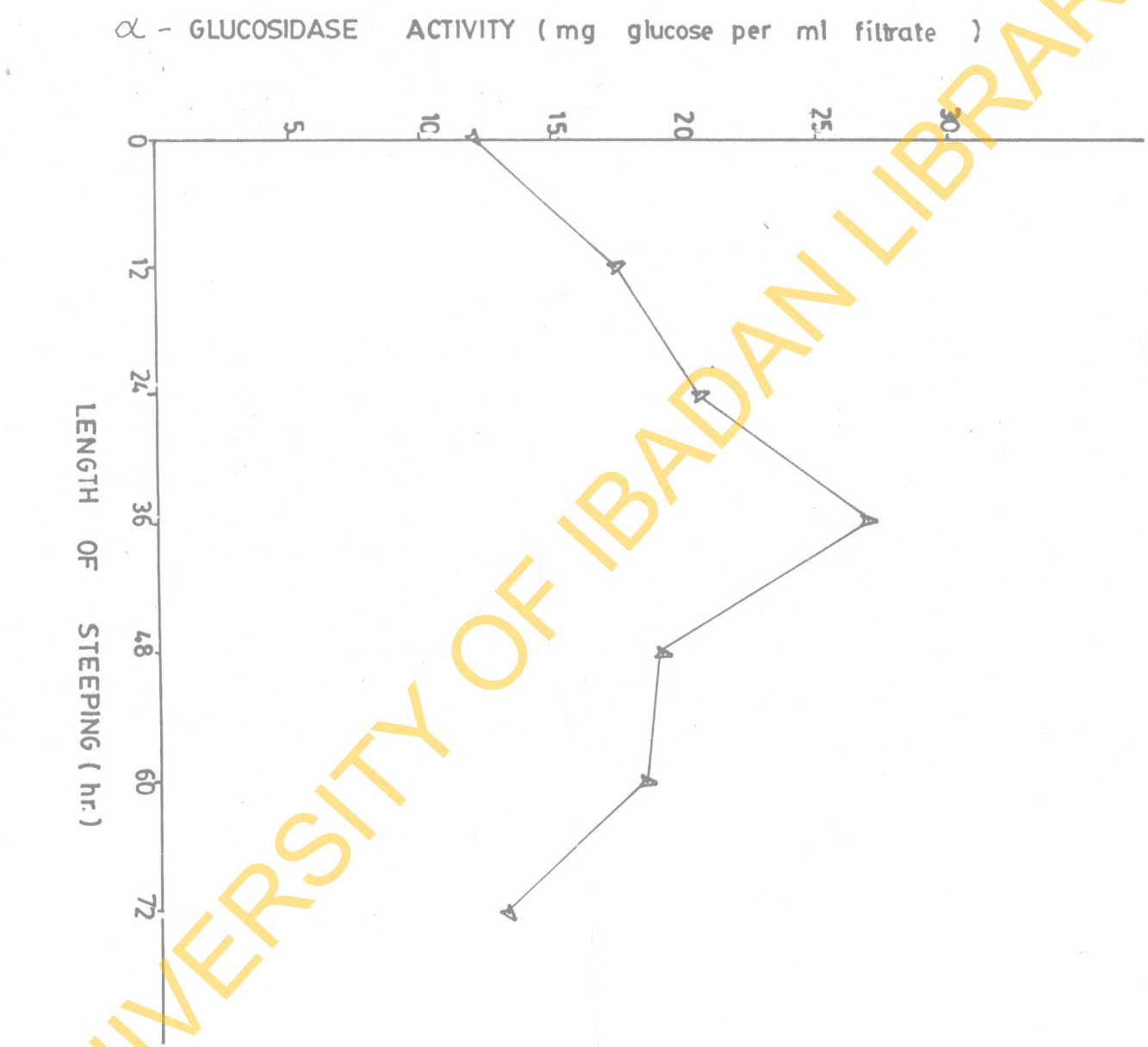


Fig. 17. Invertase activities in sorghum grain during steeping.

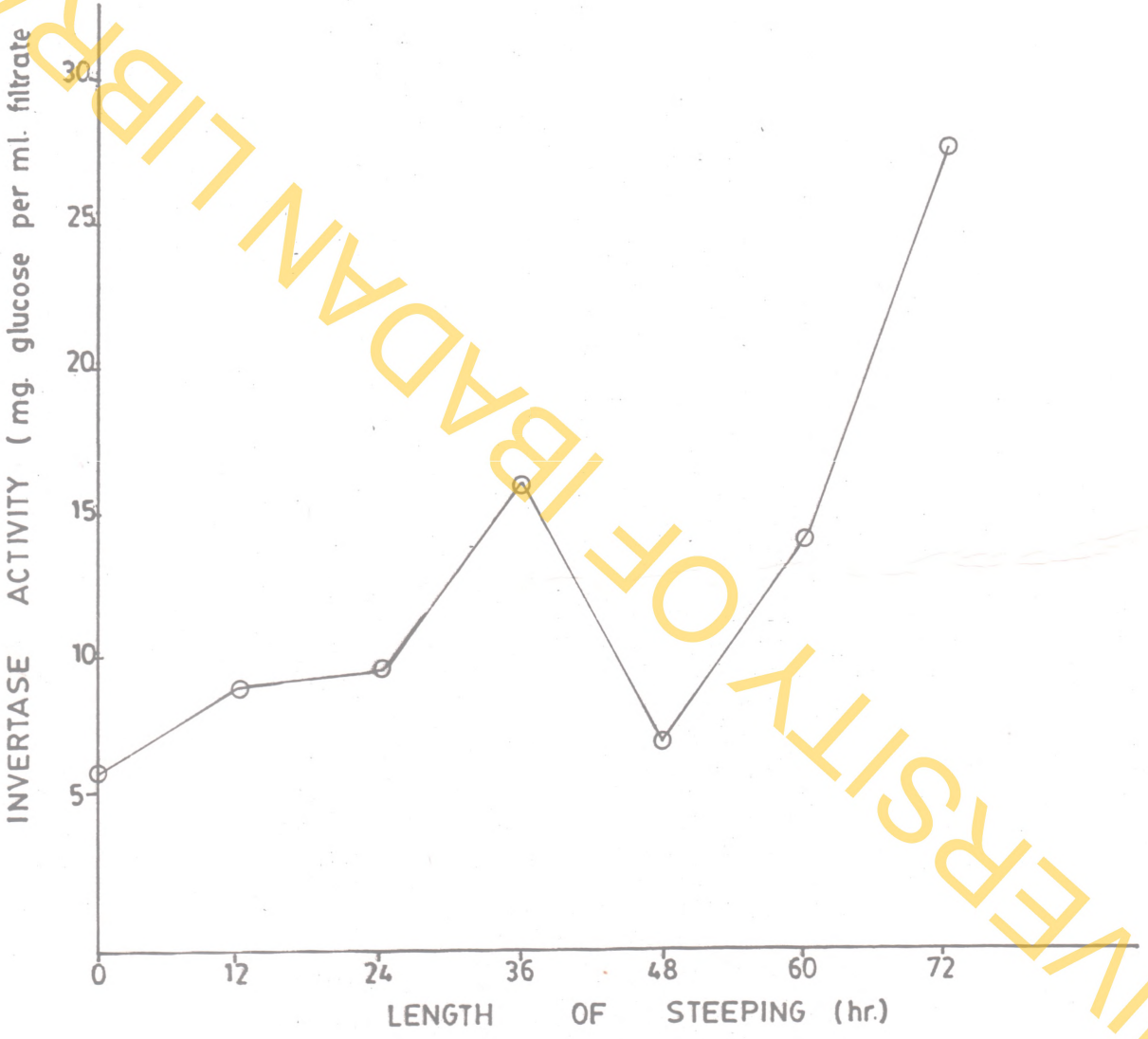


Fig. 18. Total and Alpha-amylase in isolates after 4 days.

A - S. lactis.

B - L. plantarum.

C - D. hanseniii.

D - C. krusei.

Total amylase

Alpha amylase.

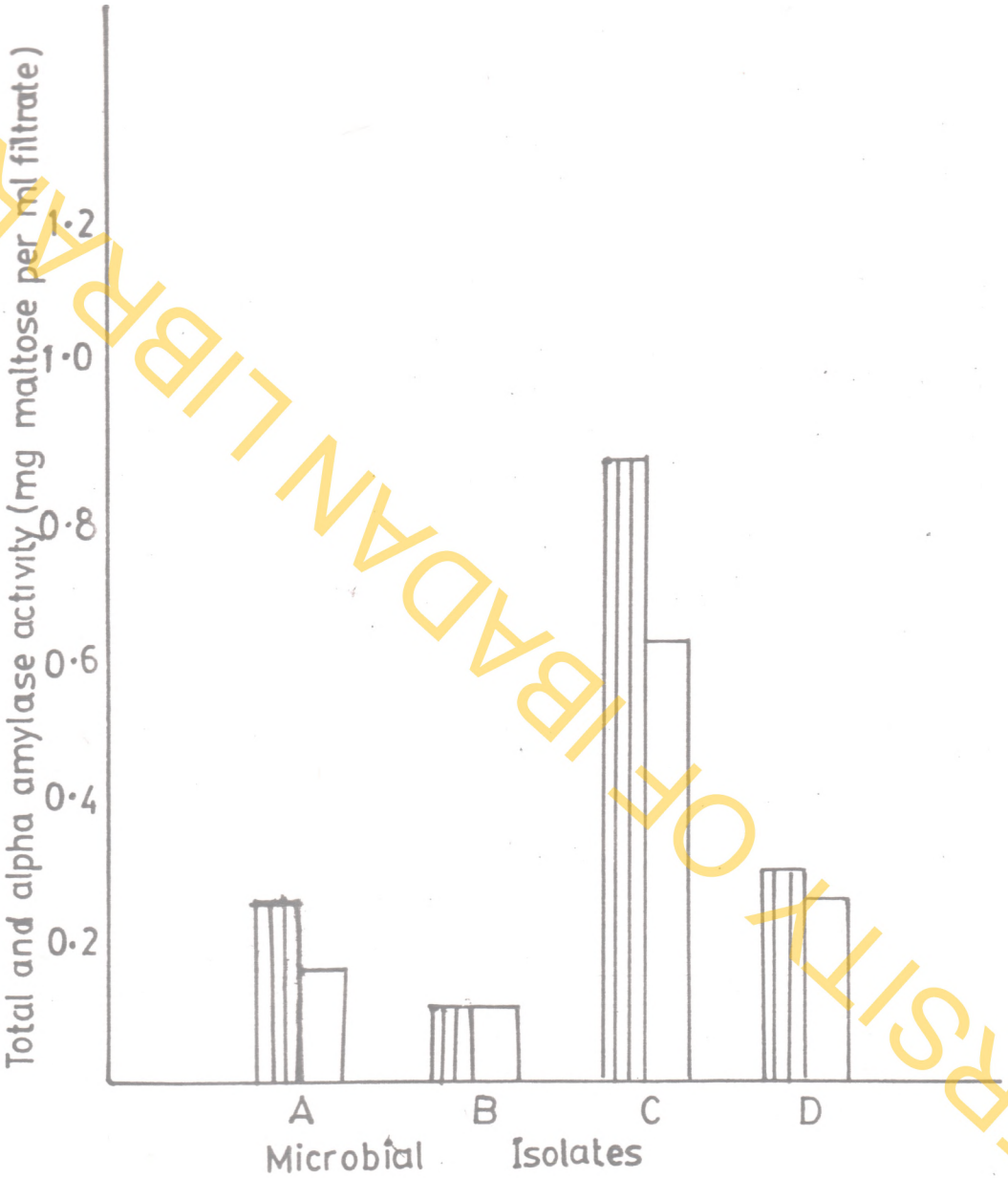


Fig. 19. Alpha-glucosidase activity in fermenting isolates after 4 days.

A - *S. lactis.*
B - *Lactobacillus* spp.
C - *L. plantarum.*
D - *D. hansenii.*
E - *C. krusei.*

Fig. 20. Invertase activity in fermenting isolates after 4 days.

A - *S. lactis.*
B - *Lactobacillus* spp.
C - *L. plantarum.*
D - *D. hansenii.*
E - *C. krusei.*

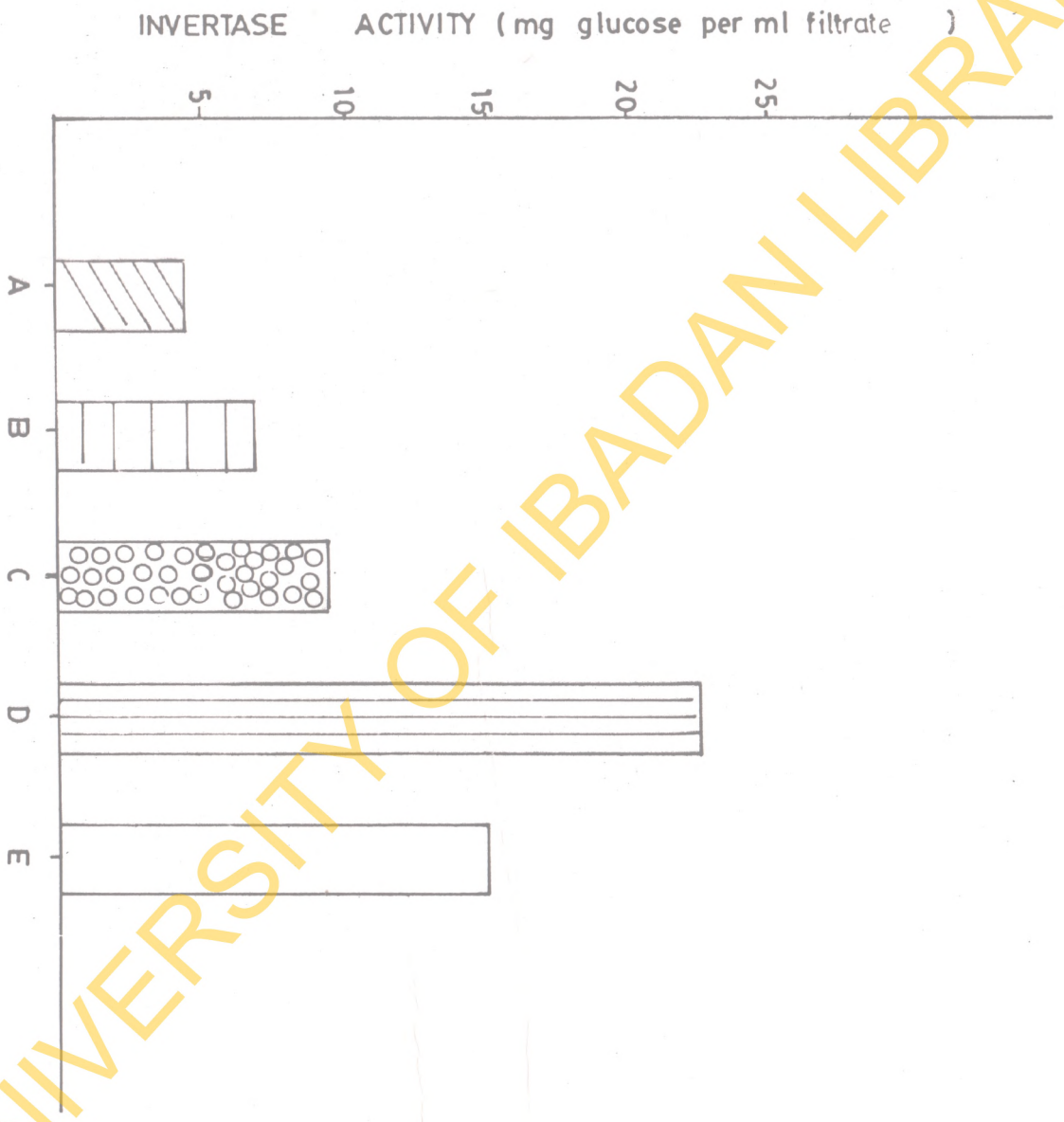


TABLE 12. Measurements of cleared zone by various bacterial isolates on starch agar plate and sugar fermentation test.

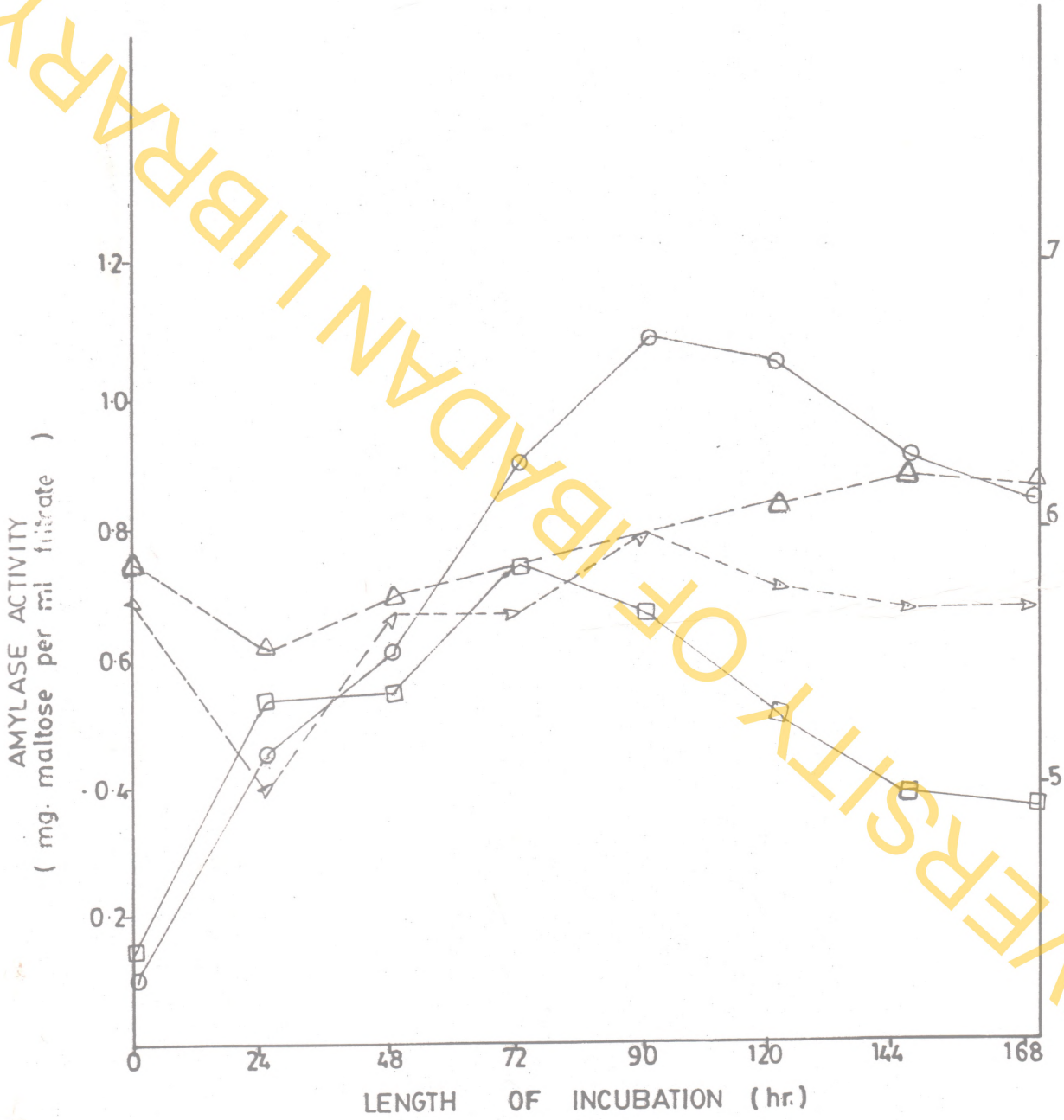
Isolate	Width of clearing	Mannitol	Xylose	Glucose	Lactose	Fructose	Sucrose	Maltose
Bacillus subtilis (1)	5cm	A	A	A	-	A	A	A
B. subtilis (2)	4.5cm	-	A	A	-	A	-	A
B. subtilis (3)	2.0cm							
Corynebacterium manihot (FIIRO strain)	3.5cm							

Key

B. subtilis (1) - Isolated from cassava.
B. subtilis (2) - Isolated from cassava starch.
B. subtilis (3) - Strain obtained from FIIRO.
A = Acid production.
- = No acid produced.

 , sorghum medium;  soluble starch medium;  ; pH sorghum medium,  , pH sol. starch medium.

Fig. 21. Time course and pH changes in the system on amylase activity in Bacillus subtilis 1.



starch (Fig. 21). For B. subtilis (2), it was 72 hr on sorghum waste and 96 hr on soluble starch (Fig. 22). Bacillus subtilis (1) produced more amylase than B. subtilis (2).

The changes in pH during growth were from acidic pH range to neutral trend for both micro-organisms (See Table 13).

At very low pH, there was little or no amylase production on sorghum waste and soluble starch by either isolate. The amylase production increased with increase in pH towards the neutral in B. subtilis (1). The optimum amylase production of B. subtilis (1) was at pH 7 in sorghum waste fermentation broth and pH 6 in soluble starch. It was same trend for B. subtilis (2) (Fig. 23).

Table 13 shows the changes in the pH of the fermentation broth after incubation. Of the different buffers employed, no activity was detected in borate buffer for the micro-organisms was found to be inhibited (Figs. 24 and 25). The highest amylase activity on the sorghum fermentation broth for both Bacillus sps. was in Tris buffer. This was followed by citrate buffer, though the activity on citrate and phosphate buffers are quite comparable. There was a shift in pH to the alkaline side in the fermentation broth of both micro-organisms at the end of fermentation (Table 14).

As shown in Fig. 26, the best temperature for the growth of B. subtilis (1) and (2) is 30^{o}C for the two media. The activity decreased with further increase in temperature.

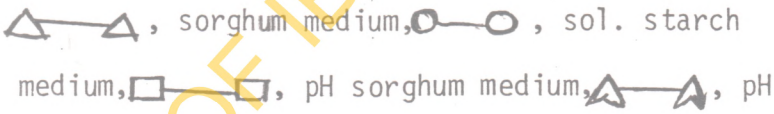


Fig. 22. Time course and pH changes in the system on amylase production in Bacillus subtilis 2.

△—△, sorghum medium, ○—○, sol. starch medium, □—□, pH sorghum medium, ▲—▲, pH sol. starch medium.

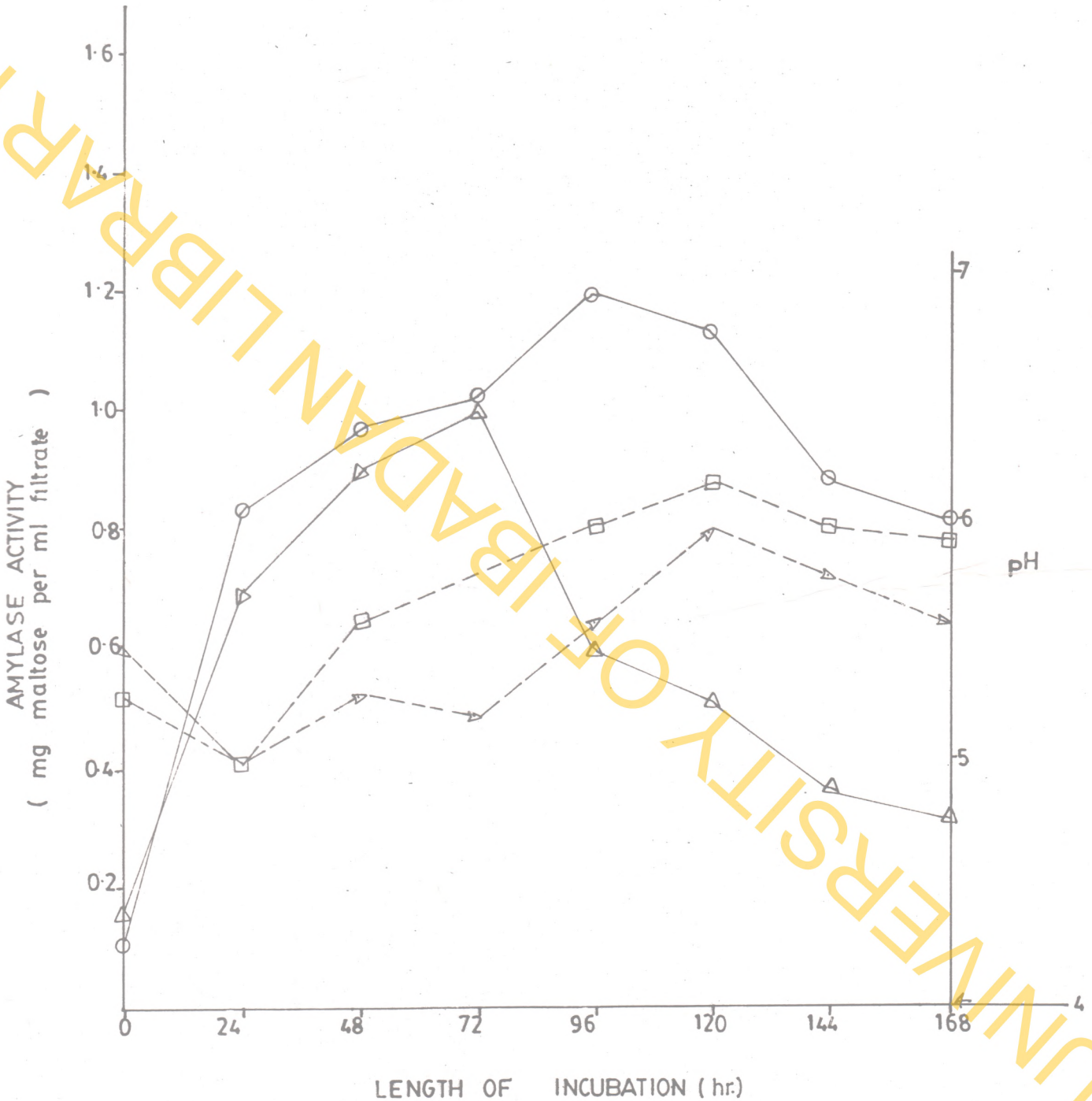
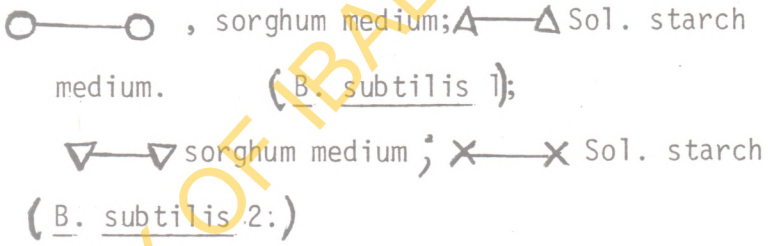


TABLE 13. pH changes of fermentation broths of both isolates after 72 hr incubation.

	B. subtilis (1)			*B. subtilis* (2)	
Initial pH	Final pH Sorghum	Final pH Sol. starch	Initial pH	Final pH Sorghum	Final pH Sol. starch
3	4.3	4.1	3	4.3	4.2
4	6.2	5.4	4	6.3	6.0
5	6.7	6.0	5	6.6	5.9
6	6.9	6.1	6	6.8	6.7
7	6.9	6.5	7	6.8	6.7
8	7.5	6.2	8	7.6	6.0

Fig. 23. Effect of pH changes on amylase production by *B. subtilis* 1 and *B. subtilis* 2.



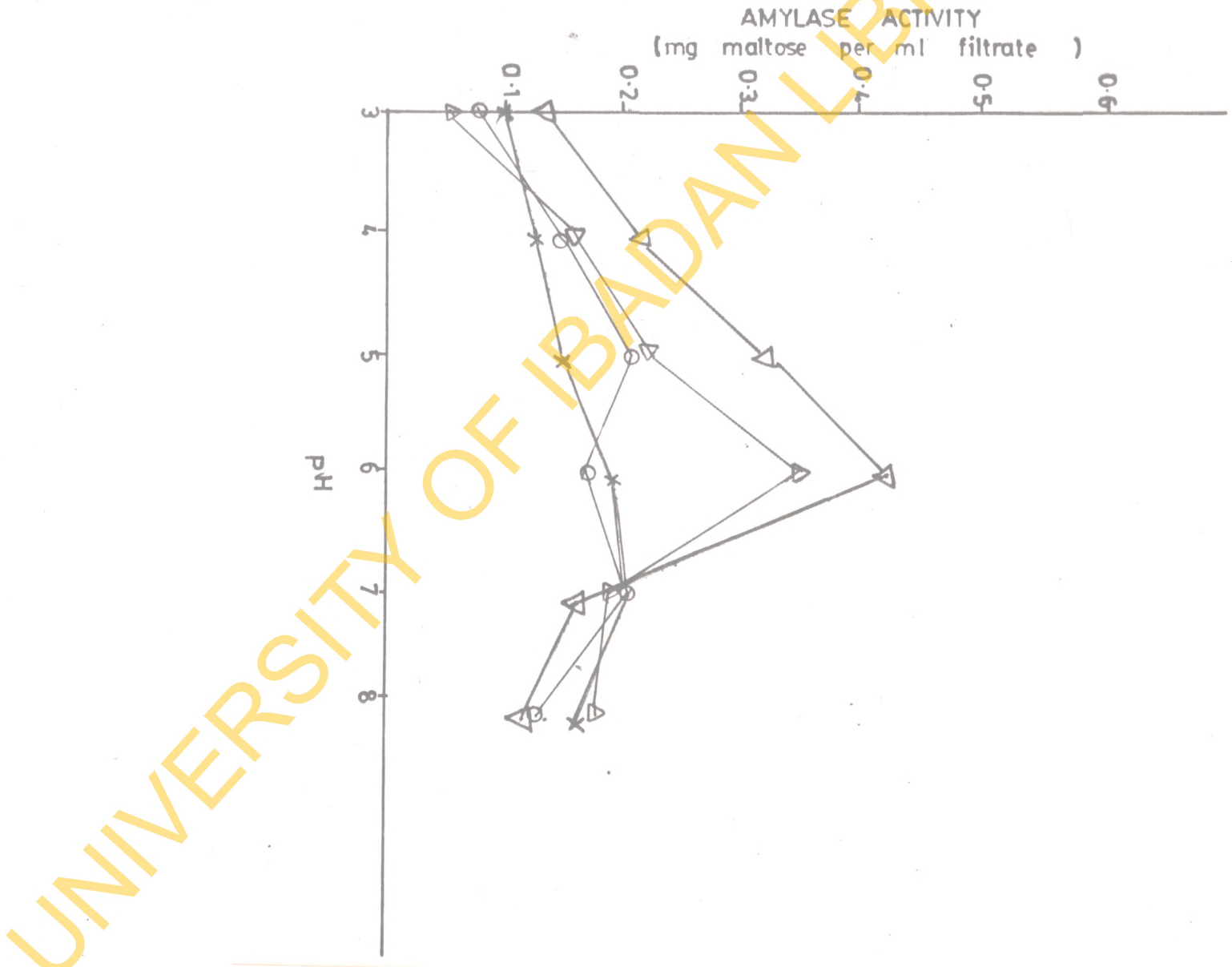


Fig. 24. Effect of buffers on amylase production (B. subtilis 1).

Sorghum waste broth

Soluble starch broth

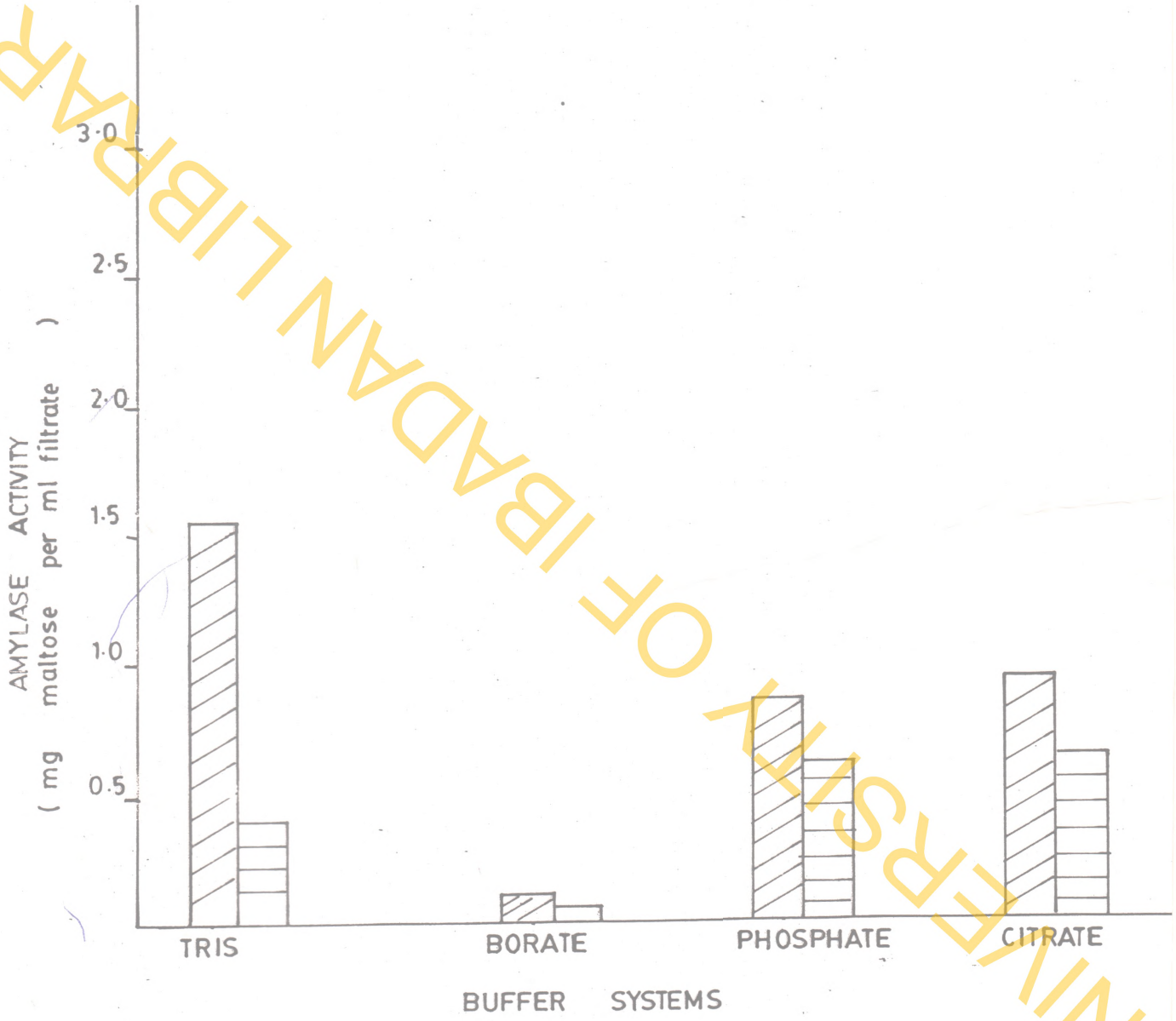


Fig. 25 Effect of buffers on amylase production (*B. subtilis* 2)

Sorghum waste broth

Soluble starch broth

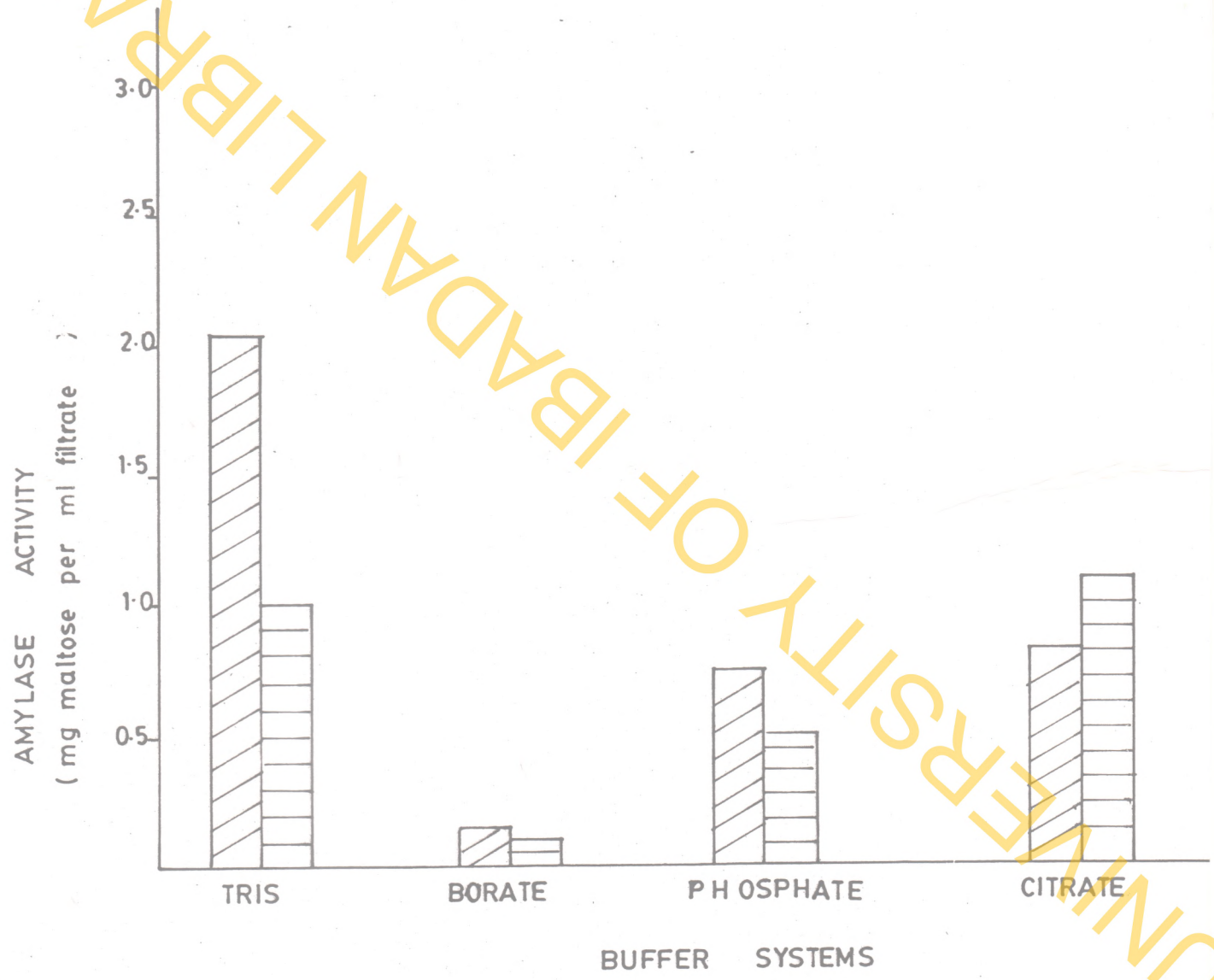


TABLE 14. pH changes (after 72 hr incubation) of different buffers on amylase production (Initial pH 6.5)

B. subtilis (1)

Buffer	Sorghum	Sol. starch
0.2M Borate	6.5	6.4
0.1M Citrate	8.2	8.3
0.2M Phosphate	6.7	6.7
0.5M Tris	8.3	8.2

B. subtilis (2)

Buffer	Sorghum	Sol. starch
0.2M Borate	6.3	6.2
0.1M Citrate	8.0	8.1
0.2M Phosphate	6.7	6.6
0.5M Tris	8.4	8.3

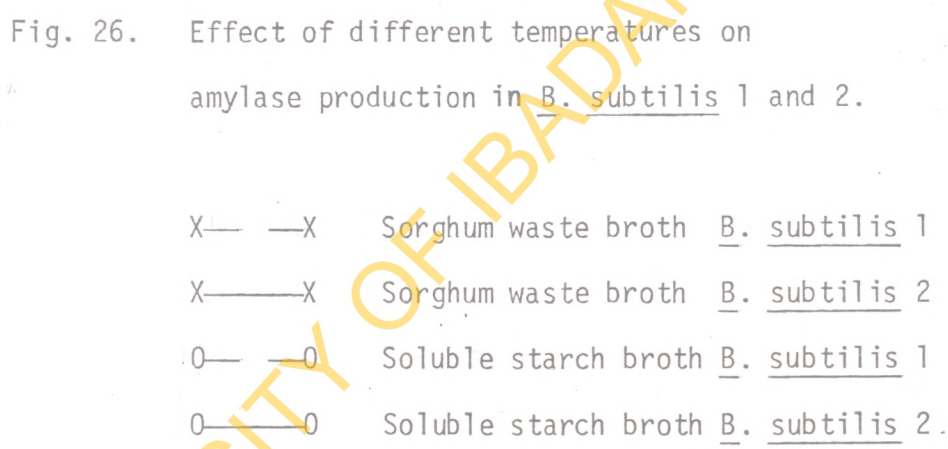


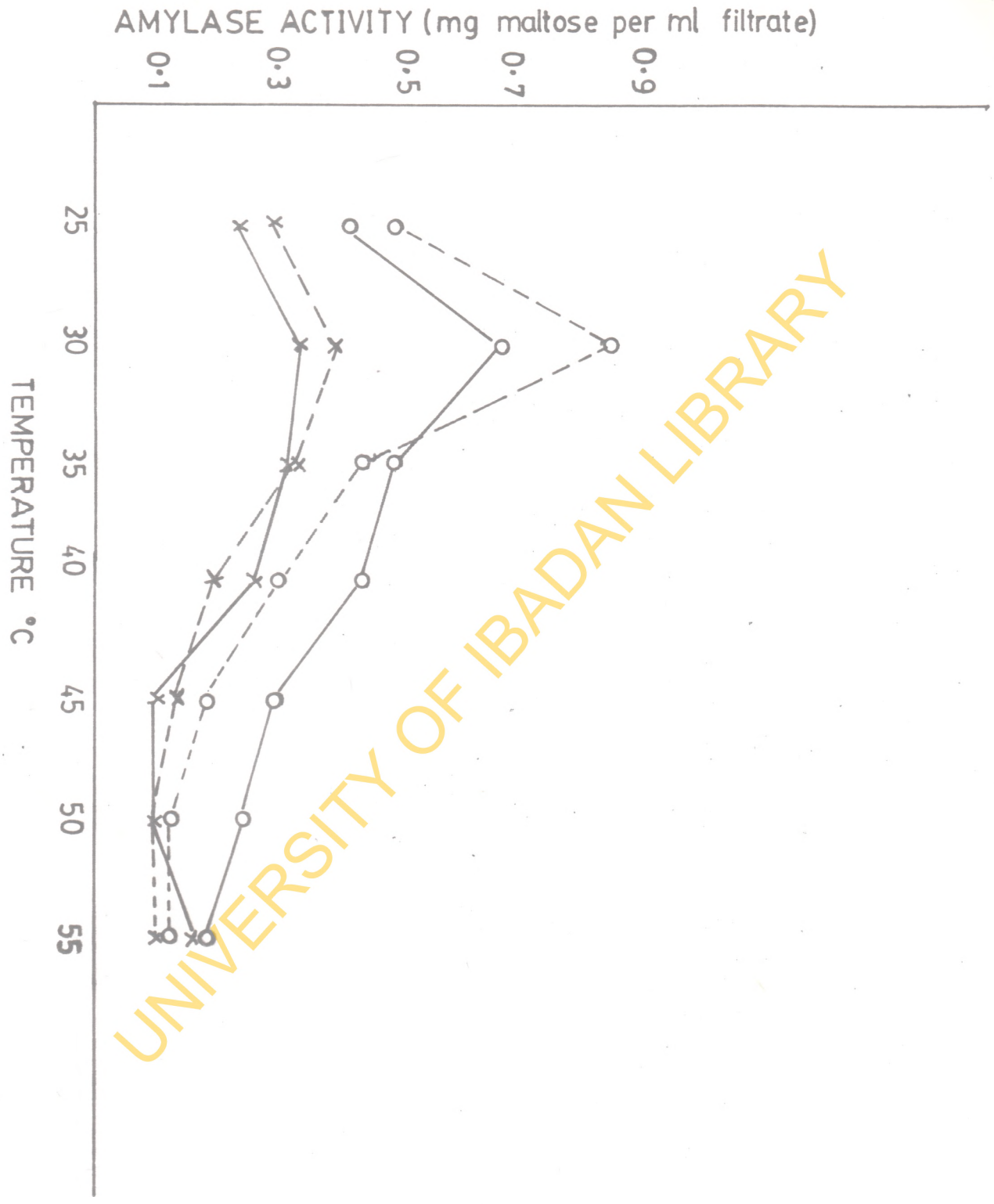
Fig. 26. Effect of different temperatures on amylase production in B. subtilis 1 and 2.

X— —X Sorghum waste broth B. subtilis 1

X——X Sorghum waste broth B. subtilis 2

O— —O Soluble starch broth B. subtilis 1

O——O Soluble starch broth B. subtilis 2.



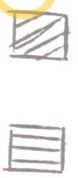
Maximum amylase production was observed when urea was used as sole nitrogen source on soluble starch medium while peptone was the best nitrogen source on sorghum waste (Fig. 27). Ammonium sulphate did not support amylase production on either sorghum or soluble starch.

In Fig. 27, it was observed that urea supported rapid growth on soluble starch medium as compared to other nitrogen sources but no growth on sorghum waste broth. Thus, the various concentrations of urea as sole nitrogen source was investigated. Figure 28 shows that there was a decrease in amount of amylase produced on soluble starch with increase in urea concentration up to 1%. However, there was an increase in amylase production in sorghum waste broth at urea concentration up to 1%. Optimal amylase activity was at pH 6.5 and 30^{o}C for both isolates (Figs. 29 and 30).

(f) Evaluation of microbial isolates for starter culture development

As shown in Fig. 31, it was observed that the sample inoculated with 24 hr steep liquor produced the highest total titratable acidity (35.1ml of 0.01N NaOH) followed by the combination of S. lactis, L. plantarum and D. hansenii. These were previously isolated from a 36 hr fermenting sample. Of the single culture inoculations, the total titratable acidities of the two yeasts were not very different. Of the lactic acid

Fig. 27. Effect of nitrogen sources on amylase production (B. subtilis 1).



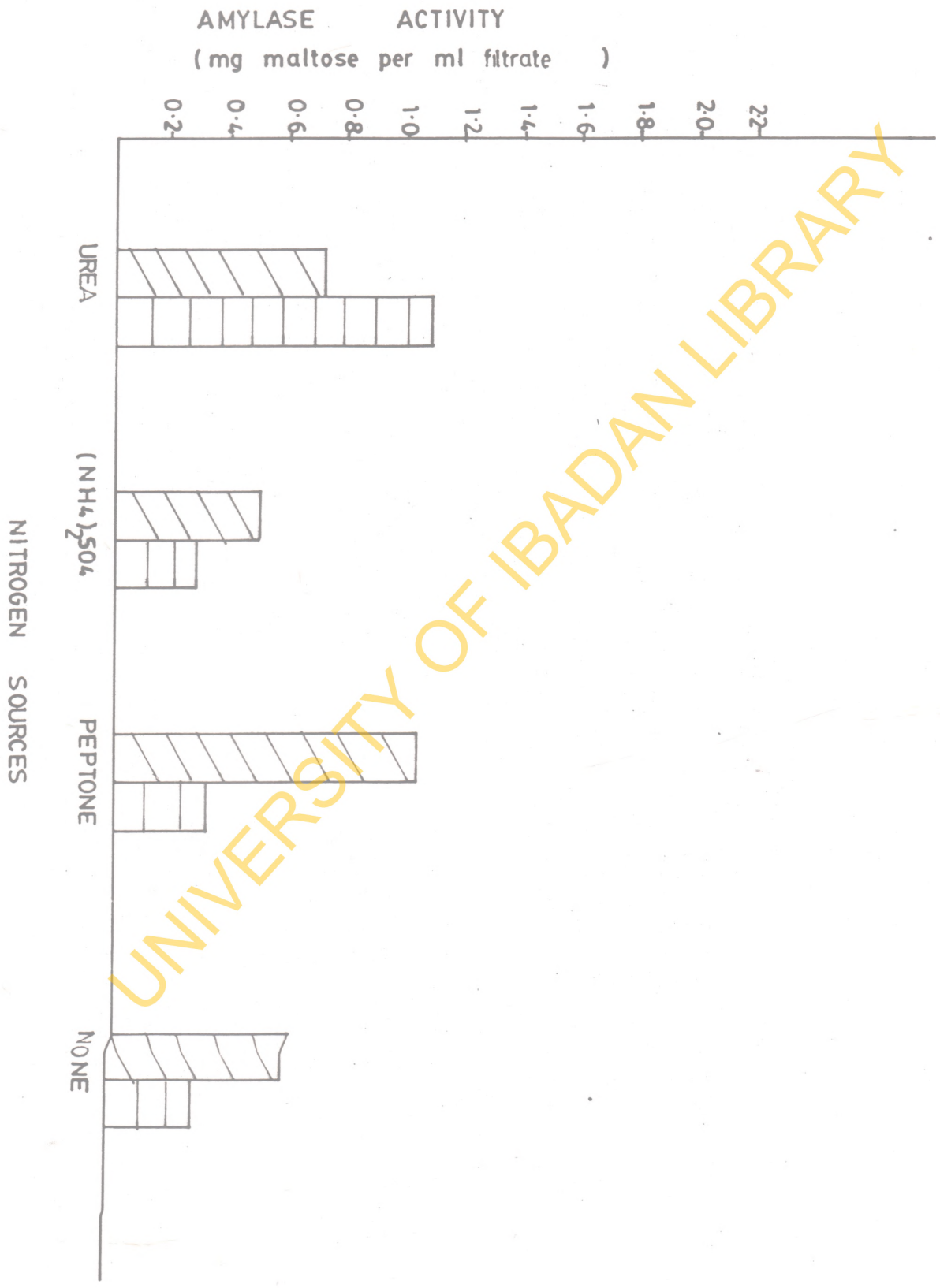
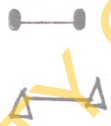


Fig. 29 Effect of temperature on amylase activity from B. subtilis 1 and B. subtilis 2.

B. subtilis 1

B. subtilis 2.

Fig. 30 Effect of pH on enzyme activity in B. subtilis 1 and 2.



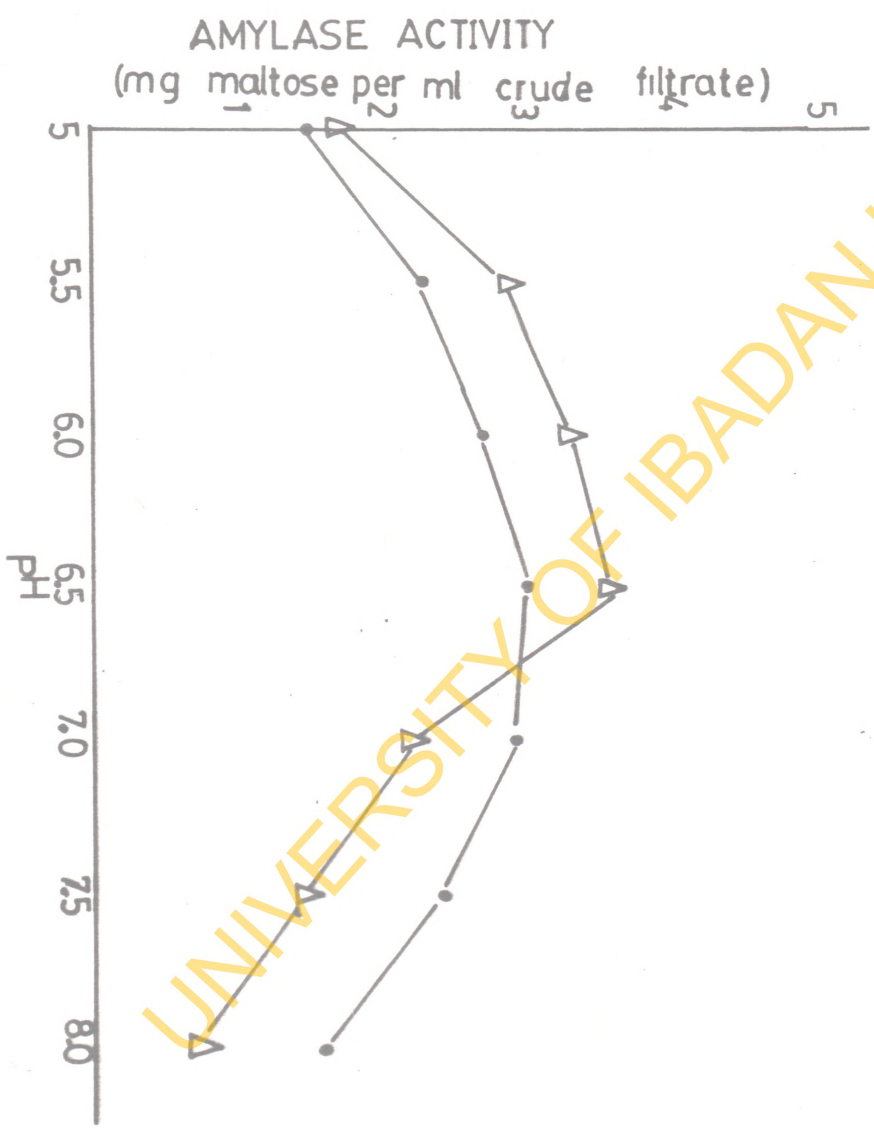
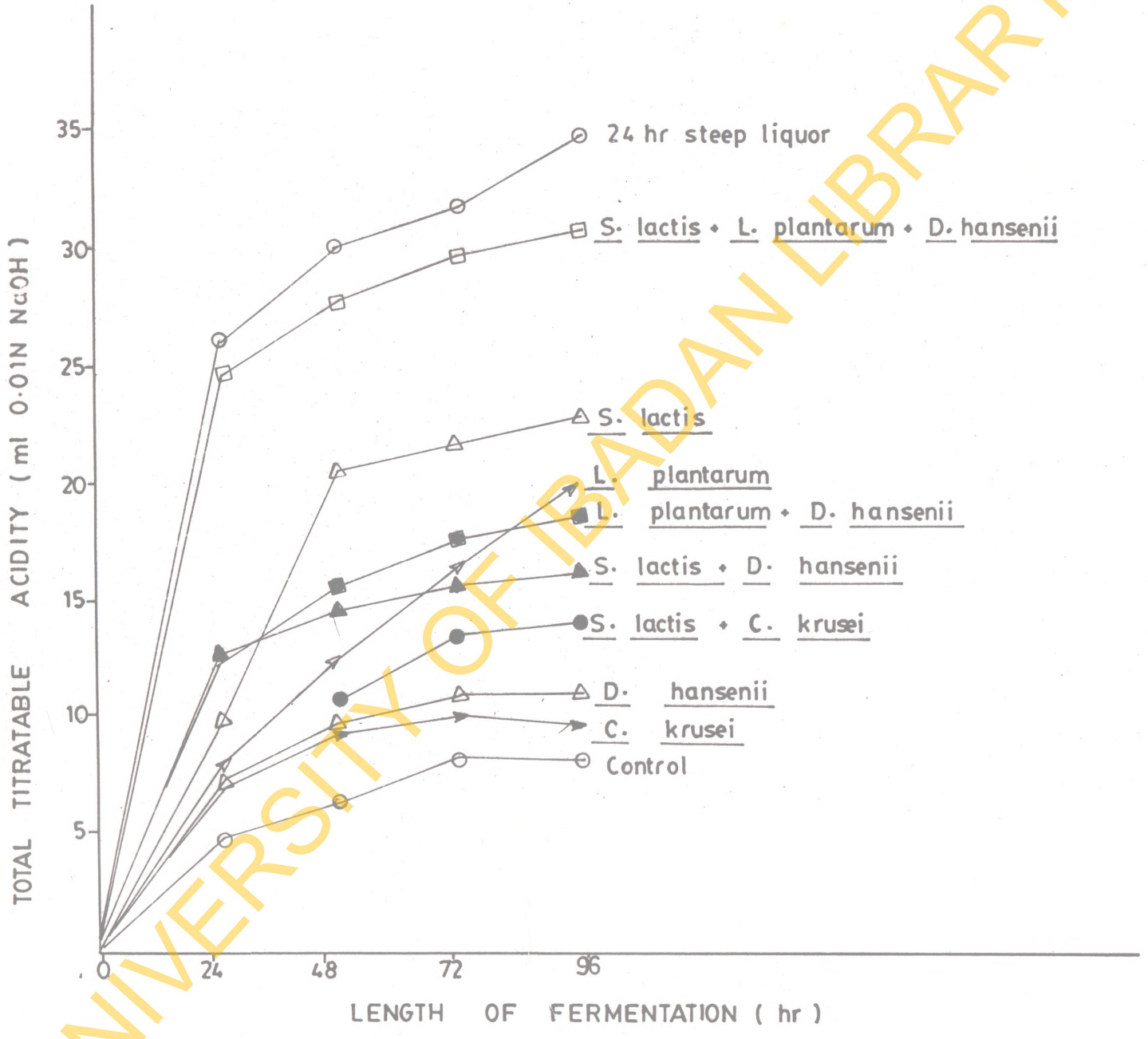


Fig. 31 Acid production by dominant micro-organisms in fermenting milled flour.



bacteria, S. lactis produced the highest acidity.

The total plate count in all the inocula increased. Streptococcus lactis increased from 8×10^4/mls to 23×10^6/ml accompanied by high acid production and a pH drop to 3.56. At the end of the fifth day, the pH of the mixed culture inoculated sample decreased from 6.7 to 3.25 (Table 15). The pH and total acidity of the mixed culture inoculated sample is similar to the locally fermented ogi-baba as shown in Table 15.

TABLE 15. Fermentation of sorghum with pure culture inoculants

DAYS	1	3	5	1	3	5	1	5
A	3.5 ± 0.1	3.2 ± 0.1	3.5 ± 0.06	10.6 ± 0.1	22.6 ± 0.1	27.5 ± 0.05	(18 ± 8) x 10^4	(23 ± 14) x 10^6
B	3.8 ± 0.05	5.5 ± 0.06	3.6 ± 0.06	9.0 ± 0.06	17.0 ± 0.01	19.1 ± 0.04	(60 ± 11)x 10^5	(41 ± 15) x 10^6
C	3.8 ± 0.00	3.7 ± 0.06	3.7 ± 0.01	7.5 ± 0.1	11.5 ± 0.05	13.3 ± 0.03	(20 ± 15)x 10^5	(100 ± 18) x 10^6
D	3.8 ± 0.1	3.7 ± 0.06	3.7 ± 0.01	7.0 ± 0.1	10.0 ± 0.1	12.5 ± 0.02	(33 ± 15)x 10^5	(45 ± 10) x 10^6
A + B	3.75 ± 0.01	3.7 ± 0.05	3.7 ± 0.01	7.1 ± 0.1	9.9 ± 0.2	13.6 ± 0.03	(33 ± 14)x 10^5	(59 ± 20) x 10^6
A + C	3.3 ± 0.01	3.4 ± 0.03	3.6 ± 0.06	12.9 ± 0.1	16.0 ± 0.1	17.5 ± 0.05	(99 ± 18)x 10^6	(110 ± 10) x 10^7
A + D	3.4 ± 0.06	3.4 ± 0.1	3.56 ± 0.06	8.0 ± 0.1	13.5 ± 0.1	14.6 ± 0.02	(55 ± 10)x 10^6	(101 ± 18) x 10^7
B + C	3.4 ± 0.05	3.4 ± 0.06	3.4 ± 0.1	16.03 ± 0.15	18.9 ± 0.05	20.1 ± 0.08	(44 ± 18)x 10^6	(44 ± 19) x 10^7
B + D	3.4 ± 0.08	3.4 ± 0.04	3.36 ± 0.15	15.1 ± 0.16	16.0 ± 0.06	16.7 ± 0.1	(11 ± 7) x 10^6	(39 ± 6) x 10^7
A + B + C	3.35 ± 0.05	3.25 ± 0.05	3.26 ± 0.11	25.0 ± 0.15	30.06 ±0.6	32.1 ± 0.1	(45 ± 14)x 10^6	(108 ± 15) x 10^7
A + B + C + D	3.4 ± 0.01	3.3 ± 0.06	3.3 ± 0.1	26.0 ± 0.06	32.0 ± 0.2	33.1 ± 0.1	(37 ± 16)x 10^6	(159 ± 28) x 10^7
24 hr steep liquor	3.35 ± 0.1	3.3 ± 0.07	3.25 ± 0.08	26.5 ± 0.1	33.1 ± 0.15	38.6 ± 0.1	(99 ± 11)x 10^4	(101 ± 31) x 10^6
Control	6.0 ± 0.07	5.9 ± 0.1	5.8 ± 0.06	5.0 ± 0.01	7.53 ± 0.06	7.6 ± 0.07	(12 ± 5) x 10^4	(14 ± 5) x 10^6

Key A = *S. lactis* C. = *D. hansenii*
B = *L. plantarum* D. = *C. krusei*

CHAPTER 4

D I S C U S S I O N

The initial surface microflora on vegetables and cereal products have been associated to be aerobic and facultative types of microorganisms such as Pseudomonas, Flavobacterium, Escherichia and Bacillus. Only few species are known to be involved with fermentation (Pederson, 1979). Akinrele (1966, 1970) studies on ogi showed that the following fungi Fusarium, Cephalosporium, Aspergillus and Penicillium were eliminated at the beginning of fermentation. He isolated Corynebacterium michiganense, Leuconostoc mesenteroides, Lactobacillus plantarum and Candida mycoderma during fermentation.

In this study of sorghum ogi, the initial surface microflora were found to be mainly fungi, L. plantarum, Streptococcus lactis and Bacillus subtilis.

The organisms isolated during the fermentation of sorghum for ogi-baba were Lactobacillus plantarum, B. subtilis, Streptococcus lactis, Pediococcus spp., Leuconostoc spp., Lactobacillus spp. and yeasts - Debaryomyces hansenii and Candida krusei. Only the B. subtilis showed diastatic activity when grown on starch agar. This is probably responsible for the conversion of the starch to

simple sugars as observed during the steeping stage. Akinrele (1966) isolated Corynebacterium michiganense as the only diastatic organism in maize preparation. Collard and Levi (1959) isolated Corynebacterium manihot in cassava, which was reported to be actively involved in the hydrolysis of cassava starch to sugars.

The predominant micro-organisms involved in the fermentation of sorghum were B. subtilis, L. plantarum, S. lactis and the yeasts. The bacteria originated from the sorghum grains as isolation showed these were present in the unfermented grains (Table 7). However, yeasts which were also found during the fermentation were not isolated from the grains or the water at the time of steeping. They are likely to come from the air or utensils used. From the previous reports, it seems that lactic acid bacteria are constantly associated with cereal grains and that the bacterial inoculum is derived from the grains. For example, the wheat flour used as a source of inoculum for mahewu (a South African sour maize meal) contains lactic acid bacteria (Hesseltine, 1979). Lactic acid bacteria were also found to be derived from the grains during commercial corn steeping (Watson et al. (1955) and soy idli fermentation (Ramakrishnan, 1979). Studies on idli, an Indian fermented black gram, rice food showed that majority of the natural flora of micro-organisms developing during the fermentation were gram positive cocci or short rods

which produces acid and gas and were identified as heterofermentative lactic acid bacteria, Leuconostoc mesenteroides and non-gas forming chains similar to Streptococcus faecalis (Mukhergee et al., 1955; Steinkraus et al., 1967).

The Penicillium and Aspergillus spp. were present on the unfermented grains most probably as contaminants. The spores of these fungal species are commonly associated with the seeds of sorghum (Tarr, 1962). The fungi do not appear to play any role in the fermentation. Their subsequent disappearance after 24 hr is probably due to the low oxygen tension in the 48 hr steeping water as fermentable microbes deplete the oxygen or it might be that the fungal species germinate and the vegetative hyphae are then killed by the anaerobic conditions.

The isolation of Leuconostoc spp. and L. plantarum from the sorghum steep liquor confirms early reports that they are one of the fermenting organisms of vegetable materials responsible for the production of biological acidity (Hucker et al., 1930; Stammer et al., 1964; Akinrele, 1966).

As the lactic acid bacteria developed in the steeping water, the acidity increased. This increase in acidity (Table 5 and 6) is probably due to the fermentation of sugars by the lactic acid bacteria. The acidity produced from sugar utilization by L. plantarum and S. lactis favoured the growth of the yeasts

which subsequently multiplied rapidly.

The association between lactic acid bacteria and yeasts in many African food and beverage fermentations is significant. It has also been observed in the food fermentations of other parts of the world (Wood, 1981). It is believed that the lactic acid bacteria provide the acid environment for growth of yeasts while the yeasts provide vitamins and other growth factors for the growth of the lactic acid bacteria (Nout, 1980; Slater, 1981).

The outcome of investigations on micro-organisms responsible for cereal fermentation precludes any generalization. Akinrele (1970) found that the predominant micro-organisms after 24 hr of steeping of maize in water were L. plantarum, Aerobacter cloaca and a Corynebacterium spp. He found that at souring stage, the micro-organisms were L. plantarum, Saccharomyces spp., Candida mycoderma and Rhodotorulla spp. Christian (1970) found only lactic acid bacteria in the fermenting maize mash. Field et al. (1981) also contended that only lactic acid bacteria were necessary for acidic cereal fermentation.

The result of this present study on sorghum gruel, ogi-baba differs from those obtained above. Lactobacillus plantarum, S. lactis and D. hansenii were predominant during steeping while S. lactis and C. krusei were the dominant micro-organisms at the souring.

Streptococcus lactis has been reported to be the main microorganism in mahewu, an indigenous sour maize beverage of Southern Africa (Hesseltine, 1979). In uji, an East Africa sour farinaceous maize beverage, the predominant fermentative micro-organism is a mixed population of lactobacilli predominantly L. plantarum (Nout, 1981). Banigo et al. (1974) produced an acceptable ogi using cultures of L. plantarum, S. lactis and Saccharyomyces rouxii. Hence S. lactis can be regarded as an important micro-organism in acidic cereal fermentations. From its acid producing properties, it probably plays the most important role in the fermentation.

The two yeasts isolated from this study, D. hansenii and C. krusei are among the species of greatest significance in foods. They have been isolated from acidic fermented foods such as olives and sauerkraut (Davenport, 1980). The role of the yeast is to impart flavour to the gruel, increase the total protein and vitamin contents.

Inoculation of single pure cultures of L. plantarum, S. lactis, D. hansenii and C. krusei into the sterile sorghum slurry did not give the required acidity for ogi-baba within a short period . (Fig. 31). The combination of cultures of L. plantarum and S. lactis inoculated into ogi slurry gave a desirable acidity through pH changes and better acidification properties (Table 15).

The inability to produce acid by pure isolated strains from a mixed culture substrates when reinoculated is not uncommon (Mbugua, 1981) particularly when the substrate has been modified for example through sterilization. The reasons for the failure could stem from varied factors such as the absence of a symbiotic partner in sterile medium which are usually present in mixed bacterial populations. The significance of this symbiotic relationship is that the lactic acid bacteria provide the acidic environment for the yeast growth while the yeast provide vitamins and other growth factors for the lactic acid bacteria (Nout, 1980; Slater, 1981).

Lactobacillus delbrueckii has been known to form much less lactic acid in sterile cereal mash. In this study S. lactis gave a higher acidification than L. plantarum (Fig. 31). Mbugua (1981) working on pure culture inoculation with uji (an East African sour maize beverage) observed that the isolated strains of L. plantarum formed relatively little acid despite its substantial growth. A substantial bacterial growth without much acid formation would appear unusual; Angeles and Marth (1971), reported that acid production is not always related to the bacterial growth rate.

The mixed culture inoculation produced acidity similar to the 24 hr steep liquor inoculation in that it improved the acidification of the ogi-baba. This is also similar to the

locally prepared ogi-baba (Table 15) in that the final acidic level is similar. The addition of a yeast, D. hansenii, improved the sample of L. plantarum inoculated ogi-baba slurry over that of S. lactis. Akinrele (1966) found that the milled sample obtained by surface sterilization of sorghum seeds with sodium metabisulphite, inoculated with the 24 hr steep liquor produced the highest acidity followed by a combination of species of Lactobacillus, Aerobacter and Saccharomyces. Banigo et al. (1974) on the other hand produced an acceptable ogi using cultures of L. plantarum, S. lactis and Saccharomyces rouxii. They implicated the yeast in the development of the flavour.

The study on starter culture evaluation showed that an acceptable ogi-baba can be obtained by priming with a 24 hr steep liquor or inoculation with a mixed culture of L. plantarum, S. lactis and D. hansenii. Secondly, results show that the mixture of organisms normally present in a fermenting mash are likely to be best suited for the fermentation of the medium.

The limitations of the method for assessing the roles of individual organism is that an organoleptic assessment was not done. This is because ethylene oxide used for sterilization might have a residual toxic effect.

The optimal growth temperature range of most of the isolates is 30 - 40^{o}C. It has been observed by various workers that the

optimal temperature for the growth of S. lactis is 30°C (Buchanan and Gibbons, 1974) or 34°C (Dohn and Rahn, 1939). Whereas Sherman (1937, 1955) observed 40°C or slightly higher as the normal growth temperature of S. lactis. Golding et al., (1943) quoted 30°C as the temperature at which lactic streptococci produce acid most rapidly.

The optimal temperature coincides with the temperature at which the fermentation takes place. It is therefore unlikely that the fermentation will be accelerated if the temperature of the fermentation mash is increased.

The unfermented sorghum grains was found to contain maltose, fructose, sucrose, glucose and raffinose (Plate 11). Previous analysis of sorghum grains by some workers have shown the presence of a similar spectrum of sugars. Von Holdt and Brand (1960) reported that fructose, glucose and sucrose were present in sorghum grains. In addition, they also recorded the presence of maltose, isomaltose, maltotriose and traces of malto-oligosaccharides. Faparusi (1970) found that ungerminated sorghum grains during burukutu production contained varying concentrations of maltose, sucrose, glucose, fructose, raffinose, maltotriose, isomaltose and stachyose. The little differences in the results obtained by the different workers have been ascribed to variations in the analytical

procedures used and in the varieties, growing and storage conditions of the grains (Gerning-Beroard, 1975).

As in previous studies, it seems sucrose is the predominant sugar in the unfermented cereal grains. Subramanian et al. (1980) analysed the soluble sugars from 10 sorghum cultivars and found that each cultivar contained stachyose, raffinose, sucrose, glucose and fructose with sucrose as the most abundant. Sucrose was also the predominant sugar in maize, wheat and corn (Gerning-Beroard, 1975; Mason and Sover, 1971; Gentinetta et al., 1979; Mbugua et al., 1983).

In this study, the oligosaccharides, stachyose and raffinose disappeared after 24 hr. The disappearance of stachyose and raffinose is significant nutritionally. These sugars are frequently associated with flatus during digestion (Rackis, 1970). The marked decrease in raffinose and sucrose shows that they are hydrolysed during the fermentation. Species of Lactobacillus and other hetero-fermentative rods produce -galactosidase which enables them to hydrolyse raffinose (Mital et al., 1973).

The initial fall in the concentration of sugars at 12 hr and the subsequent increase (Fig. 9) during the steeping could be as Faparusi (1970) explained. During the steeping, sorghum grains imbibe water and hence trigger off metabolic activities of the grains during which some of the sugars will be used for these

metabolic activities. However, when subsequently the enzymatic activities increase more sugars will be produced through amylolytic activities than are required for metabolism. During the fermentation of sorghum for producing a Sudanese bread, kisra, the total and non reducing sugars were also found to decrease markedly at the commencement of fermentation (Tinay et al., 1979). The subsequent increase in sugars could also be due to autoamylosis by the endogenous alpha and beta-amylases in the grain. Auto-amylosis of uncooked maize grits in water lead to an increase in reducing sugars (Nout, 1980).

The decrease in sugars other than glucose on the chromatogram is remarkable (Plates 11 and 12). Bond and Glass (1963) noted a decrease in sucrose and increase in glucose content of dent corn on steeping for 2 days; the decrease was assumed to be due to the fact that sucrose was preferentially used as a respiratory substrate during early stages of growth initiation. The sucrose decreased significantly in the first 24 hr hence it seemds to be the major fermentable sugar. Sucrose was also confirmed as the major fermentable substrate in maize fermentation for uji production (Rackis et al., 1970). The evidence in this sorghum steeping study is that its sucrose was hydrolysed to glucose and fructose by the enzyme invertase which was found in the fermenting grains. The

decrease in fructose in the chromatogram might be due to its utilisation by the yeasts.

Edelman et al. (1959) have presented evidence that sucrose was synthesised in the scutellum of cereal seedlings by a mechanism involving uridine diphosphoglucose. One could therefore expect that a mechanism like this could lead to the formation of glucose in sorghum. This is not evident in the chromatogram in that L. plantarum and S. lactis utilise glucose (Fig. 11 and 12). However, the glucose concentration during the steeping as evident by the colour intensity in the chromatogram is fairly constant.

In the fermenting sample, it could be expected that fermentable sugars especially monosaccharides could be quickly used up. The sugar utilization as determined by the pH and titratable acidity changes showed that some microorganisms utilise sugars more than others. The differences in microorganism utilization of some specific sugars are attributable to varying levels of enzymes that convert some sugars to glucose or acids (Stern et al., 1977). The sugars are also converted from one form to the other at different stages of the fermentation, thus affecting the sequence of occurrence of these microorganisms during the fermentation period.

Streptococcus lactis and L. plantarum also utilised maltose (Table 10). These two probably contained some levels of maltase to break down the maltose in the grain into their constituent

monossacharides of glucose and galactose. The utilization of sucrose by S. lactis and and the two yeasts demonstrates different levels of invertase present: the enzyme breaks down the sucrose to fructose and glucose. This could also account for the disappearance of sucrose in the fermenting grains by hydrolysing it to glucose and fructose.

The sugars that were produced enzymatically during the fermentation also supported good growth of the fermentative microorganisms (Table 10). Also the sugars which were present originally in the unfermented grains and decreased during the fermentation were observed to be utilised by the dominant fermenting microorganisms.

The reduction in the sugar level during souring is presumably due to the utilization of sugars by the lactic acid bacteria and yeasts for acidification. At the souring stage, all the sugars were used for acid production.

The result of Figure 15 shows that after 24 hr of steeping of whole sorghum grains in tap water, amylase activity fluctuated. The α-amylase dropped within the first 12 hr and subsequently remained stable with the control.

It was claimed by Bond and Glass (1963) that α and β-amylase were not present in the resting seed of maize and that it was only on the 6th day of steeping and growth initiation that α-amylase

was secreted and became predominantly active. Akinrele (1966) observed no ∝-amylase during his study on maize and concluded that the importance of steeping for ogi is to soften the kernel and build up a predominant microflora of lactic acid bacteria. However, in this study, it was observed that there was no ∝-amylase activity in the unsteeped sorghum grains.

It has been shown by various workers that resting kaffir corn (sorghum) grains contained no ∝-and B-amylase (Dyer and Noveille, 1966; Daiber and Noveille, 1968) but are formed in the grains only in the course of germination (Kneen, 1944). However, in the case of ogi-baba, the sorghum grains steeped in water do not germinate and this could account for absence of amylase activity during the preparation.

Studies on enzyme activities during steeping showed that sorghum grains contained invertase and ∝-glucosidase. These two enzymes were active up to 36 hr before showing a decrease (Figs. 16 and 17). The glucose produced from the enzymatic activities could be responsible for the proliferation of lactic acid bacteria. The lactic acid bacteria could also have produced some ∝-glucosidase. Chan and Li (1981) reported intracellular ∝-glucosidase in Lactobacillus acidophillus NCTC 1973.

Debaryomyces dekkeri and Candida utilis were listed by Davies (1963) amongst the yeasts that possess invertase. A review

of this by Nueberg and Roberts (1946) showed the invertase in yeasts to be an important activity in the conversion of common carbohydrate sources to assimilable sugars. In this study, Debaryomyces hansenii produced the highest invertase activity (Fig. 20) which might be responsible for decrease of sucrose as observed in the chromatogram.

From these results, a general inference that can be made on enzymes activity during ogi from sorghum is that both the microbial components, notably the amylolytic Bacillus sp., the yeasts and the cereal enzymes are active in saccharifying the grains. Akinrele (1966) isolated Corynebacterium michiganense as the only diastatic organism in maize preparation for ogi. However, during the preparation of ogi-baba, the Bacillus subtilis was the main amylolytic bacterium and it probably initiated the saccharification. Subsequently, the cereal and microbial invertase and -glucosidase which were the main enzymes detected during the fermentation, continued the saccharification. The yeasts produced the highest invertase (Fig. 20).

The result given in figures 21 and 22 showed that during the process of attaining optimal amylase production on the ogi-baba processing waste broth, the hydrogen ion concentration changed gradually towards neutral pH. This shows that buffers will be of great importance in formulating an appropriate fermentation

media as it will eliminate abrupt changes in the hydrogen ion concentration. As shown in figures 24 and 25, there was no activity on borate buffer. This might be due to the microbial inhibitory activity of borate. It is pertinent to note that boric acid is extensively used in pharmaceutical products as an antiseptic against the growth of certain bacteria and fungi (The British Pharmaceutical Codex, 1949). Comparing citrate and phosphate buffers, it was observed that citrate gave higher amylase activity than phosphate. Tris buffer gave the highest activity (Figs. 24 and 25). The reason for the high activity in media with Tris and citrate buffer can be attributed to the shift of pH value to the alkaline side, i.e. pH 6.5 to pH 8.4. Such alkalinity is within the optimum recorded by Mahmoud et al. (1967) and Hoogerheide (1954) who stated that maximum potency was reached at pH 8.5 after which gradual decrease in potency occurred. Phosphate buffer on the other hand has a high buffering capacity thus keeping the pH unchanged (Table 14). Another reason why citrate promoted amylase activity is perhaps because it can be used as a carbon source by some bacteria such as B. subtilis. For this reason, the final pH of citrate waste medium is an indication of citrate utilization by some micro-organisms.

Regarding the optimum temperature of growth of the strain used, it was found that the maximum enzyme production was at $30^{o}C$

(Fig. 26). Wallestein (1937) is of the opinion that best results for enzyme production were obtained when incubation temperature is the same as the temperature for growth. This could be so since each micro-organism had its own optimum temperature.

Of the various nitrogen sources utilised, urea favoured enzyme production on soluble starch medium (Fig. 27). Taha et al. (1967) observed that the corn starch had some ingredients which enhance the activity of amylase enzyme. The result in this study showed that amylase activity increased with urea concentration up to a particular level of concentration (1%) after which, with increase in urea concentration there was a decrease in activity (Fig. 28). High concentration of urea has been known to have some denaturing effect on enzyme activity and this may be due to its marked effect in increasing the intrinsic viscosity of enzyme protein (Hopkins, 1946).

From these results, in order to further optimize the production of ogi-baba, some areas need further investigation. The amount of sugars during the steeping and souring could be determined more precisely by gas chromatographic method. This will reveal sugars present in trace amounts. Also more work is needed on the development of the starter cultures. This includes the determination of appropriate drying methods to produce the microbial inocula and the correct inoculum ratio. Subsequently, the starters could

be produced as dehydrated powders and distributed in Sachets for use by ogi-baba producers.

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Appendix A - Identification of yeasts.

NATIONAL COLLECTION OF YEAST CULTURES

Cat. No. | Species Debaryomyces hansenii | Strain 1 | Alt. Cat. No. C606

Deposited by Dr S.A. Odunfa, University of Ibadan, Nigeria | Date November 1983

Isolated by

SOURCE Fermenting guinea corn

INFORMATION

REFERENCES

MORPHOLOGY

Cells:	
Broth 48 hr	Round to oval, single, but mainly in groups (3-7) × (4-9)μ
Agar 48 hr	Round to long oval, single (2-5) × (2-7)μ
Culture:	
Broth 21 d	Forms a non-flocculent deposit; no film or ring
Agar 21 d	Pinkish cream, shiny, smooth.
Plate cultures:	PDA, CMA – ie 21 days
Pseudo/true mycelium	

Asexual spores	Ballistospores	Arthrospores	Endospores
	–	–	–

Sexual spores:	+ KAc, YM
Conjugation	–
Ascus	◎◎
Ascospores	round, smooth, 1 per ascus

CAT No. SPECIES *Debaryomyces hanseni* C606

FERMENTATION

-ve 21 days

Glucose		Inulin	
Galactose		Starch	
Maltose		Melezitose	
Sucrose		Trehalose	
Lactose		∝-methyl-D-glucoside	
Melibiose		Cellobiose	
Raffinose		Xylose	

ASSIMILATION OF CARBON COMPOUNDS 21 days

Glucose	+	Lactose	−	L-arabinose	+	Ribitol	+	Succinic acid	+w
Galactose	+	Melibiose	+	D-arabinose	−	Galactitol *	+	Citric acid	+
Sorbose	+	Raffinose	+	D-ribose	+	D-mannitol	+	Inositol	−
Sucrose	+	Melezitose	+	L-rhamnose	+	D-sorbitol	+	Gluconolactone	+
Maltose	+	Inulin	−	Ethanol	+	α-methyl glucoside	+	Glucosamine	−
Cellobiose	+	Soluble starch *	−	Glycerol	+	Salicin	+	Methanol	−
Trehalose	+	D-xylose	+	Erythritol	+	Lactic acid	+L		

ASSIMILATION OF NITROGEN COMPOUNDS

Ammonium sulphate	+
Ethylamine hydrogen chloride	−
Cadaverine	NT
Potassium nitrate	−

* differs from standard description

MISCELLANEOUS

Hydrolysis of fat	−	21 days	Growth in vitamin-free medium	−	21 days
Acid production	−	"	Growth with 100ppm cyclohexamide	tw	"
Growth at 37°C	−	"	Growth with 1000ppm cyclohexamide	−	"
Hydrolysis of arbutin	+	14 days	Growth with 50% glucose	+	14 days
Urease activity	−	21 days	Growth with 60% glucose	−	21 days
Starch production	−	"			

Fermentation pattern in EBC tall tube test

Growth with 10% NaCl + "
" " 15% " + "
" " 20% " − "

NATIONAL COLLECTION CULTURES

Cat. No. Species Candida krusei Strain 2 Alt. Cat. No. C607

Deposited by Dr S. A. Odunfa, University of Ibadan, Nigeria Date November 1983

Isolated by

SOURCE fermenting guinea corn

INFORMATION

REFERENCES

MORPHOLOGY

Cells:

Broth 48 hr	Long to elongated in pairs and chains (4-5)x(6-17)μ
Agar 48 hr	" " " single and in chains (3-6)x(6-14)μ
Culture:	
Broth 21 d	Forms a milky climbing film and a non-flocculent deposit.
Agar 21 d	Cream, dull, rough
Plate cultures: Pseudo/true mycelium	PDA aer – well developed pseudomycelium with irregular branches and oval blastospores at verticils. anaer – as above but only few branches. CMA aer –ve anaer well developed pseudomycelium, no branches, interspersed with elongated cells.

Asexual spores	Ballistospores	Arthrospores	Endospores
	–	–	–

Sexual spores:	–
Conjugation	
Ascus	
Ascospores	

CAT No. SPECIES Candida krusei CB07

FERMENTATION

Glucose	+	2 days	Inulin	—	21 days
Galactose	—	21 days	Starch	—	"
Maltose	—	"	Melezitose	—	"
Sucrose	—	"	Trehalose	—	"
Lactose	—	"	α-methyl-D-glucoside	—	"
Melibiose	—	"	Cellobiose	—	"
Raffinose	—	"	Xylose	—	"

ASSIMILATION OF CARBON COMPOUNDS 21 days

Glucose	+	Lactose	—	L-arabinose	—	Ribitol	—	Succinic acid	—
Galactose	—	Melibiose	—	D-arabinose	—	Galactitol	—	Citric acid	—
Sorbose	—	Raffinose	—	D-ribose	—	D-mannitol	—	Inositol	—
Sucrose	—	Melezitose	—	L-rhamnose	—	D-sorbitol	—	Gluconolactone	+w.h
Maltose	—	Inulin	—	Ethanol	+	α-methyl glucoside	—	Glucosamine	—
Cellobiose	—	Soluble starch	—	Glycerol	+	Salicin	—	Methanol	—
Trehalose	—	D-xylose	—	Erythritol	—	Lactic acid	+		

ASSIMILATION OF NITROGEN COMPOUNDS

Ammonium sulphate	+
Ethylamine hydrogen chloride	—
Cadaverine	NT
Potassium nitrate	—

MISCELLANEOUS

Hydrolysis of fat	—	21 days	Growth in vitamin-free medium	+	7 days
Acid production	—	"	Growth with 100ppm cyclohexamide	—	21 days
Growth at 37°C	+	7 days	Growth with 1000ppm cyclohexamide	—	"
Hydrolysis of arbutin	—	21 days	Growth with 50% glucose	—	"
Urease activity	—	"	Growth with 60% glucose	—	"
Starch production	—	"	Fermentation pattern in EBC tall tube test		
			Growth with 10% NaCl	—	"

Appendix B - Publication.

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Microbiological Changes During the Traditional Production of *ogi-baba*, a West African Fermented Sorghum Gruel

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The micro-organisms responsible for the spontaneous acidic fermentation in *ogi-baba*, a fermented sorghum gruel, were investigated. The *ogi-baba* was produced in the traditional way. The micro-organisms present in the unfermented grains at the initial stage of steeping were various species of *Aspergillus* and *Penicillium* fungi as well as bacteria of the following genera – *Bacillus*, *Lactobacillus* and *Streptococcus*. As the steeping progressed, the microbial population consisted of *Lactobacillus*, *Streptococcus*, *Leuconostoc* and a yeast, *Debaryomyces hansenii*. Of these, *Lactobacillus plantarum* and *Streptococcus lactis* were predominant. At the souring stage. *S. lactis* and another yeast, *Candida krusei*, were the dominant micro-organisms. Throughout the fermentation the titratable acidity increased, thereby resulting in a gradual fall in pH. The dissolved oxygen during the steeping also fell from 28% saturation to 2·1% saturation within 48 h.

Introduction

The common substrates for fermentation in tropical Africa are cassava and cereal grains, such as maize, sorghum and millet. The cereal grains are fermented and milled to produce thin gruels and alcoholic beverages which are known by various names in different parts of West Africa[1].

In the Yorubaland of Nigeria, this fermented, thin gruel is called *ogi* and can be prepared from fermented maize (*Zea mays*), Guinea corn (sorghum) (*Sorghum bicolor*) and millet (*Pennisetum typhoideum*). When it is prepared from sorghum it is called *ogi-baba*. *Ogi* is a sour, fine-paste beverage. In this respect it differs from other African fermented beverages such as *mahewu* and *uji*, which are farinaceous and gritty gruels.

Ogi-baba is one of the most important fermented cereals in West Africa. This is so since sorghum, commonly called Guinea corn, is the largest cultivated cereal crop in Nigeria and other West African countries. The Nigerian Federal Office of Statistics (1966) estimated that about 2·6 million tons were produced in the 1965/66 season from parts of Northern Nigeria. Sorghum contains more protein and tannin but lower soluble carbohydrate, oil and mineral matter than maize[2]. Only high tannin varieties are cultivated in tropical Africa, because of their resistance to depredation by birds[3]. The production of *ogi-baba* is a traditional domestic art, done with rudimentary equipment. The fermentation is spontaneous and uncontrolled and, hence, results in a product of variable quality.

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Various workers have carried out studies on the fermentation of maize for *ogi* production[4–8]. A similar maize gruel from Ghana was also studied by Andah and Muller[9]. However, there is no information on the micro-organisms responsible for fermentation of sorghum for *ogi-baba*. Knowledge of the microbiology is essential for the development of starter cultures, which are prerequisites for commercial manufacture of these products. In this paper, the microbiological changes during fermentation of sorghum are reported.

Experimental

Materials and methods

The traditional method of sorghum *ogi* was followed: sorghum (2 kg) was soaked in tap water (5 l) for 1–3 days at room temperature (28 ± 2 °C). The steep water was then decanted off and the steeped grains were wet-milled in a commercial-scale Burr mill and sieved through a fine wire-mesh screen to remove the pomace, which was discarded. The filtrate was allowed to settle for 1–2 days (souring stage) to produce a sediment (*ogi-baba*), recovered by decanting the supernatant liquor.

Isolation procedure

Samples (10 ml) of steep liquor were removed each day under aseptic conditions, and serially diluted. The 10^{-6} and 10^{-7} dilutions were plated out. One set of plates was incubated aerobically at 30 °C and a duplicate set was incubated at 30 °C in a gas jar under an anaerobic atmosphere, of H_2 and CO_2 provided by standard BBL (Baltimore Biological Laboratories) disposable generators. After 24 and 48 h, the colonies developing on the plates were counted. All micro-organisms within a sector of each plate were isolated and examined microscopically and for gross morphology.

Media

Several media were used to isolate the various micro-organisms occurring in the fermenting sorghum. The media used were malt agar for yeasts and moulds, potato dextrose agar containing streptomycin (30 µl/ml) for moulds and yeasts[10], Difco's APT[11] and Man, Rogosa and Sharpe (MRS) agar[12] for lactic acid bacteria and plate count agar for total viable counts. For subsequent work and subculturing, MRS agar was used for lactic acid bacteria and malt agar for yeasts and moulds.

Characterisation of isolates

Characterisation of isolates employed microscopic examination and conventional physiological and biochemical tests[13].

Cell morphology was examined using 24-hour-old cultures. The aerobic/anaerobic relationships of the isolates were investigated in tubes of yeast extract semi-solid agar (in which the dissolved oxygen had been previously boiled out) by stabbing with a needle down the length of tubes which were then incubated for 5 days at 30 °C. Aerobic organisms grew only at the surface and the facultative anaerobes grew throughout the length of the tube. The anaerobes grew only at the base of the tube.

The biochemical tests carried out on all isolates were (i) catalase activity, (ii) ability to hydrolyse starch, and (iii) utilization of sugars. Proteolysis was assessed in tests using litmus milk, gelatin liquefaction and casein hydrolysis[13].

The semi-solid medium of Gibson and Ab-del-Malek[14] was used to determine the hetero- and homo-fermentative nature of the lactic acid bacteria.

Dissolved O_2 in the steep liquor was measured at 24 h intervals using a YSI (Yellow Springs Instrument Corporation) model 53 oxygen electrode.

Identification of micro-organisms

Identification of the microbial species was by reference to standard methods[15, 16]. The identification of the yeasts was confirmed at the National Collection of Yeast Cultures, Norwich, U.K.

Results

Table I shows the variation in the number of micro-organisms associated with the different stages of the fermentation during steeping. The results show that the microbial population was mainly bacteria and yeasts. Fungi occurred only on the unfermented grains. During steeping, *Lactobacillus* and *Streptococcus* species appeared initially and a yeast, identified as *Debaryomyces hansenii*, appeared after 48 h and these organisms constituted the main microbial population throughout the later stages of steeping. The mould count had decreased sharply by 24 h of steeping. The yeasts appeared on the second day and persisted until the end of souring. In the souring process, the major organisms were found to be *Streptococcus lactis* and another yeast, identified as *Candida*

TABLE I. Microbiological changes during the steeping stage of sorghum fermentation

(a) Total counts (per ml) during aerobic incubation

Time (h)	Lacto-bacilli[a]	Total lactics[b]	Yeasts[c]	Count on PCA[d]	Moulds
0	$6{\cdot}2 \times 10^4$	$1{\cdot}0 \times 10^5$	—	$8{\cdot}9 \times 10^6$	$2{\cdot}0 \times 10^2$
24	$2{\cdot}5 \times 10^5$	$1{\cdot}2 \times 10^6$	—	$2{\cdot}0 \times 10^8$	—
48	$1{\cdot}5 \times 10^6$	$4{\cdot}0 \times 10^8$	$9{\cdot}3 \times 10^2$	$1{\cdot}5 \times 10^9$	—
72	$5{\cdot}0 \times 10^7$	$4{\cdot}4 \times 10^9$	$7{\cdot}5 \times 10^4$	$2{\cdot}4 \times 10^9$	—

(b) Total counts (per ml) during anaerobic incubation

Time (h)	Total Lacto-bacilli[a]	Total lactics[b]	Total count on PCA
0	$7{\cdot}1 \times 10^4$	$1{\cdot}8 \times 10^5$	$9{\cdot}2 \times 10^5$
24	$1{\cdot}9 \times 10^6$	$2{\cdot}3 \times 10^6$	$2{\cdot}3 \times 10^6$
48	$1{\cdot}2 \times 10^7$	$0{\cdot}9 \times 10^7$	$1{\cdot}2 \times 10^7$
72	$4{\cdot}7 \times 10^7$	$1{\cdot}2 \times 10^7$	$1{\cdot}1 \times 10^7$

Data represent means of three replicate determinations.

a Out of 15 *Lactobacillus* spp. isolated, 13 were identified as strains of *Lactobacillus plantarum*

b Out of 22 Lactics isolated, nine were *L. plantarum*, six were *Streptococcus lactis*, four were *Pediococcus* sp.

c Out of 12 yeasts isolated, 10 were strains of *Debaryomyces hansenii*

d Out of 31 isolates on PCA, eight were *L. plantarum*, 15 were *Bacillus subtilis*, five were *S. lactis*, five were *Pediococcus* sp. and five were *Enterobacteriaceae*.

TABLE II. Microbiological changes during souring stage of sorghum fermentation.

Total plate counts (per ml) during aerobic incubation

Time (h)	Total Lacto-bacilli[a]	Total lactics[b]	Total yeasts[c]	Total count on PCA[d]	Total moulds
0	$4{\cdot}2 \times 10^8$	$1{\cdot}8 \times 10^7$	$2{\cdot}0 \times 10^6$	$1{\cdot}3 \times 10^9$	—
12	$4{\cdot}5 \times 10^8$	$2{\cdot}0 \times 10^8$	$3{\cdot}1 \times 10^6$	$1{\cdot}7 \times 10^9$	—
24	$6{\cdot}3 \times 10^8$	$2{\cdot}3 \times 10^9$	$5{\cdot}4 \times 10^6$	$1{\cdot}9 \times 10^9$	—
36	$7{\cdot}2 \times 10^8$	$2{\cdot}5 \times 10^9$	$9{\cdot}7 \times 10^6$	$2{\cdot}0 \times 10^9$	—
48	$8{\cdot}0 \times 10^8$	$2{\cdot}7 \times 10^9$	$1{\cdot}2 \times 10^6$	$2{\cdot}4 \times 10^9$	—

(b) Total counts (per ml) during anaerobic incubation

Time (h)	Total Lacto-bacilli	Total lactics	Total count on PCA
0	$7{\cdot}1 \times 10^6$	$1{\cdot}0 \times 10^7$	$1{\cdot}2 \times 10^6$
12	$8{\cdot}9 \times 10^6$	$1{\cdot}3 \times 10^7$	$1{\cdot}3 \times 10^6$
24	$1{\cdot}1 \times 10^7$	$4{\cdot}7 \times 10^7$	$1{\cdot}7 \times 10^6$
36	$1{\cdot}3 \times 10^7$	$7{\cdot}9 \times 10^7$	$1{\cdot}9 \times 10^6$
48	$2{\cdot}1 \times 10^7$	$2{\cdot}7 \times 10^8$	

Data represent means of three replicate determinations.

a Out of 12 *Lactobacillus* spp. isolated, 10 were strains of *L. plantarum*.

b Out of 17 lactics isolated, eight were *Streptococcus lactis*, two were *L. plantarum*, three were other *Lactobacillus* spp. one was *Leuconostoc* sp.

c Of the 21 yeasts isolated 18 were strains of *Candida krusei*.

d Of the 28 isolates on PCA five were identified as *Bacillus subtilis*, seven as *S. lactis*, one as *L. plantarum* and five as *Enterobacteriaceae*.

krusei. Table II shows the microbial count during the subsequent souring process. The results show that, of the total viable bacteria, counts for both *Lactobacillus* spp. and for total lactic acid bacteria increased gradually over 48 h.

The unfermented grains contained bacteria of the following genera, *Bacillus*, *Lactobacillus* and *Streptococcus*, and members of two fungal genera, *Aspergillus* (mostly *A. niger*) and *Penicillium*. Viable fungal species could not be detected after steeping the grains for 24 h. The biochemical characteristics of the representative isolates of the yeasts are shown in Table III. *Candida krusei* is cream-coloured with well-developed pseudomycelium but without spores. It is capable of fermenting and assimilating glucose. *Debaryomyces hansenii* is pinkish-cream with no mycelium and possesses ascospores. It is capable of assimilating many carbon sources. It differs from the type species in its ability to assimilate galactitol but not soluble starch.

Tables IV and V show the pH changes and acidity values during steeping and souring.

TABLE III. Morphological and biochemical characteristics of the yeasts of fermenting sorghum

	Laboratory reference no.	
	S015	S020
Colony morphology	Cream, dull, rough on 48 h agar plate	Pinkish cream, shiny, smooth on 48 h agar plate
Cell shape and size	Long to elongated, singie and in chains (3–6) × (6–14) μm	Round to oval, single but mainly in groups (2–5) × 2–7) μm
Pseudomycelium	Well-developed on PDA with irregular branches and oval blastospores at verticils	
Sporulation	–	Round, smooth ascospores, 1 per ascus
Fermentation		
Glucose	+	+
Galactose	–	–
Maltose	–	–
Sucrose	–	–
Lactose	–	–
Mellibiose	–	–
Raffinose	–	–
Assimilation		
Glucose	+	+
Galactose	–	+
Maltose	–	+
Sucrose	–	+
Lactose	–	+
Mellibiose	–	+
Raffinose	–	+
Soluble starch	–	–[a]
Galactitol	–	+[a]
Oxygen relationship	Facultative anaerobe	Facultative anaerobe
Yeasts identified	*Candida krusei*	*Debaryomyces hansenii*

[a] Differs from standard description.

The titratable acidity (expressed as percentage lactic acid) increased throughout the steeping and souring, thereby resulting in the gradual fall in pH. During the steeping, the dissolved oxygen in the water decreased substantially, from 28% of O_2-saturation level in the (air-saturated) water initially, to 2% saturation after 48 h.

TABLE IV. Changes in pH and acidity during the steeping stage of sorghum fermentation

Time (h)	pH	Titratable acidity (% lactic acid)	Dissolved oxygen (% saturation)
0	6·4	0·30 ± 0·04	28·0
24	6·0	0·45 ± 0·03	12·0
48	5·6	0·51 ± 0·05	2·1
72	5·0	0·53 ± 0·06	2·0

Each figure is a mean of three replicate determinations (±S.D.)

TABLE V. Changes in pH and acidity during the souring stage of sorghum fermentation

Time (h)	pH	Titratable acidity (% lactic acid)
0	5·0	0·54 ± 0·01
24	4·8	0·62 ± 0·04
48	3·8	0·78 ± 0·05

Each figure is a mean of three replicate determinations (±S.D.)

Discussion

The predominant micro-organisms involved in the fermentation of sorghum were bacteria (predominantly *Lactobacillus* and *Streptococcus*) and yeasts. The bacteria originated from the sorghum grains, as isolation and analysis showed that these were present in the unfermented grain during steeping (Table I). However, yeasts which were also found in increasing amounts during the fermentation were not isolated from the grains nor from the water at the time of steeping. From previous literature, it seems, in general, that lactic acid bacteria are associated with cereal grains and that the bacterial inoculum for natural fermentation process is derived from the grains. For example, the wheat flour, used as a source of inoculum for *mahewu* (a South African sour maize meal), contained lactic acid bacteria[17]. Lactic acid bacteria were also found to be derived from the grains during commercial corn steeping[18] and *soy idli* fermentation[19].

Fungi of *Penicillium* and *Aspergillus* spp. were present as contaminants on the unfermented grains. The spores of these fungal species are commonly associated with the seeds of sorghum[3]. However, the fungi do not appear to play any role in the fermentation, and their subsequent disappearance after 24 h is probably due to the low oxygen tension in the steeping water.

As the lactic acid bacteria proliferated in the steeping water, the acidity increased, presumably due to the fermentation of sugars and the acidity that developed favoured the growth of yeasts, which subsequently multiplied rapidly.

The association between lactic acid bacteria and yeasts is common in many food and

beverage fermentations[20]. It has been suggested that the yeasts and lactic acid bacteria are in symbiotic association, the lactic acid bacteria providing the acid envirnoment for yeast growth, whilst the yeasts provide vitamins and other growth factors for the lactic acid bacteria[21]. Unpublished information from this laboratory has shown that both *D. hansenii* and *C. krusei* are favoured by acid production; the pH optima for their growth are 6 and 5, respectively.

Investigations on the micro-organisms responsible for acidic cereal fermentations in other African foods, have shown a range of species; for example Akinrele[4] found that the predominant micro-organisms after 24 h of steeping maize in water were *Lactobacillus plantarum*, *Aerobacter cloaca* and a *Corynebacterium* sp. He found that, at the souring stage, the micro-organisms present were *L. plantarum*, *Saccharomyces* spp., *Candida mycoderma* and *Rhodotorula*. Christian[22] found only lactic acid bacteria in fermenting maize mash. Fields *et al.*[8] also contend that only lactic acid bacteria were necessary for the fermentation of corn meal.

The picture presented by the results of the present study of sorghum gruel (*ogi-baba*) differs from the above examples in the microbial composition. *Lactobacillus* spp., *Streptococcus* spp. and the yeast *D. hansenii* were predominant during steeping, whilst *S. lactis* and a yeast, *C. krusei*, were the dominant micro-organisms at the souring stage. The difference in the microbiological composition from fermenting maize may be ascribed to differences in the chemical composition between maize and sorghum, particularly the presence of tannins in sorghum. Tannins have been found to inhibit the growth and fermentation of some micro-organisms, including *Saccharomyces cerevisiae*[23] and hence could influence the microbial balance in the fermenting mash.

Streptococcus lactis is reported to be the main micro-organisms in *mahewu*, an indigenous sour maize beverage of Southern Africa[17]. In *uji*, an East African sour farinaceous maize beverage, the predominant fermentative agent is a mixed population of lactobacilli, especially *L. plantarum*[24]. Banigo *et al.*[7] produced an acceptable *ogi* using cultures of *L. plantarum*, *Streptococcus lactis* and *Saccharomyces rouxii*. Hence, *Streptococcus* spp. and *Lactobacillus* spp. can be regarded as dominant micro-organisms in acidic cereal fermentations, their importance being due to their acid-producing properties[25, 26].

The two yeasts isolated from *ogi-baba* are among the species of greatest significance in foods. Whereas, in most instances, the proliferation of these yeasts in foods, such as fermented olives and sauerkraut, is undesirable[27], they make a useful contribution in some other foods, such as fermented cereal gruels, in which they improve the flavour acceptability[4] and increase the total protein and vitamin contents by their biomass. *Candida krusei* has been isolated more frequently from fermenting plant substrates, especially acidic fermentations. For example, it was found to be the predominant yeast in the fermentation of *busaa*, a Kenyan traditional opaque maize beer[28], and in the fermentation of apples in India[29]. Twenty-seven strains of *C. krusei* were also isolated from sourdough starter cultures[30].

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