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REPORT ON VISIT TO CNRST IRSAT DEPARTMENT OF FOOD TECHNOLOGY

(DTA) OUAGADOUGOU, BURKINA FASO

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BY

MARY HALM (MISS)
FOOD RESEARCH INSTITUTE (CSIR)
ACCRA, GHANA

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By Mary Halm

Objectives

To conduct analysis of Soumbala production at the Pilot plant from raw material to final product and determine composition of dominating micropopulations at genus level;

to isolate representative and dominating *Bacillus* species from intermediate and final product and maintain them at 4^0 C as slope cultures on Nutrient Agar;

to determine pH and a_w values of samples from different stages of fermentation and

to implement Good Laboratory Practice (GLP) at DTA.

Microbiological analysis of Soumbala production

Stages involved in production of Soumbala

Soumbala is produced at the Pilot Plant from *Parkia biglobosa* seeds. The seeds are first cleaned by winnowing and removal of chaff and foreign matter. They are then thoroughly washed and boiled in pots for 24 hours. The boiled seeds are drained in baskets and pounded in a mortar to dehull. After dehulling, sorting and washing they are boiled for 2 to 3 hours and again drained in baskets. They are then packed in baskets and a small quantity of millet flour sprinkled on and mixed with the seeds before covering with a sack. The mixture is allowed to ferment for two days after which the fermented seeds are dried in solar dryers or on plastic sheets for two or mores days depending on the intensity of the sun. The dried seeds are milled into a fine powder and packed in portions of 100 g in polythene bags ready for sale and subsequent use as a condiment or a source of protein.

Two batches of production were examined. The first batch started from Tuesday 17 th June 1997 and ended on Monday 23 rd June 1997. The samples collected for analysis included : raw seeds; millet flour; dehulled seeds after second boiling; dehulled seeds plus millet flour at the beginning of fermentation; after 24 hours fermentation; after 48 hours fermentation; dried material after two days fermentation and the milled and packaged product.

The second batch of fermentation started on Tuesday 24 th June 1997 and ended on Monday 30 th June.

Microbiological Analysis

Ten grams of each sample were taken and 90 ml sterile diluent containing 0.1 % peptone, 0.85 % sodium chloride, pH 7.0, were added and homogenised in a stomacher (Lab Blender Model 4001, Seward Medical) at normal speed for 30 seconds. From appropriate tenfold dilutions , colony forming units were determined by pour plate method on Plate Count Agar (DIFCO 04796- 17 Lot 97876 JB) incubated at 30° C for 3 days for aerobic mesophiles; de Man Rogosa & Sharpes (MRS) Agar (DIFCO 0882-17-0, Lot 95960 JA Detroit, USA) incubated anaerobically in an anaerobic jar with anaerocult A at 30° C for 5 days for lactic acid bacteria; Malt Agar with added gentamycin (40 mg per litre) incubated at 30° C for yeasts and moulds.

Isolation of Dominating micro-organisms

All colonies totalling about 20 to 30 from the highest dilution or from a sector (more than 15%) of the plate were picked into the corresponding broth and streaked onto the agar substrate until pure cultures were obtained.

Characterisation of Isolates

Isolates from PA were subcultured in Nutrient Broth , streaked on PCA plates and examined for colony and cell morphology, Gram reaction, catalase production and oxidase tests. Bacillus species were recognized as Gram-positive, catalase-positive oxidase-negative/positive rods with or without phase bright spores.

Isolates from MRS Agar were subcultured in MRS Broth and streaked onto MRS Agar.

Pure cultures were examined by colony and cell morphology, Gram reaction , catalase production, oxidase test and Hugh and Leifsons test (Hugh and Leifson, 1953).
Colonies on MA were identified by colony and cell morphology.

Gram reaction was carried out by mixing a loopful of the culture in 3% KOH and examining whether it was slimy or not. Gram positive reaction was indicated when the mixture was not slimy and slimy reaction indicated Gram negative reaction. For catalase production a loopful of culture was mixed with a drop of 3 % hydrogen peroxide on a microscope slide and observed for gas bubbles to indicate production of catalase.

Oxidase tests were conducted using strips of oxidase paper.

Hugh and Leifsons test was conducted by inoculating two tubes containing O/F Medium (MERC 10282, Damstadt, Germany) plus 1 % glucose (Glucose Monohydrate MERC 1.08342) with each organism and topping one tube with paraffin oil to obtain anaerobic condition. The tubes were incubated at 30° C for 2 to 7 d. Fermentative reactions were indicated by yellow coloration in both anaerobic and aerobic tubes due to acid formation. Oxidative reactions were indicated by yellow

coloration in the top section of the aerobic tube only and no colour change in the anaerobic tube.

pH determinations

For all samples, 10 g were taken and mixed with 10 ml distilled water and the pH determined with a pH meter (HANNA Instruments 8520, France).

a_w determinations

Water activity measurements were taken with an a_w meter (a_w Wert Awx 3001 ebro) by placing a filled cup of the sample in the chamber for a minimum of two hours until the reading is stabilised.

Results and observations

Results of pH and a_w measurements and microbial counts on PCA , MRS and MA obtained from the two batches of production are presented in Tables 1 and 2.

There was great variation in the numbers of bacteria observed on both the PCA and MRS plates between the two batches of production examined. However, after dehulling and second boiling of the seeds and addition of millet flour, the fermentation began with an average count of 3.4×10^4 c.f.u./g on PCA and 4.9×10^2 c.f.u./g on MRS plates. A significant increase was observed after 24 h fermentation for both batches of fermentation and maximum counts of more than 10^8 c.f.u./g was obtained. This was followed by a slight increase (a factor 10) after 48 h fermentation when the fermentation was stopped.

Isolations were made from the PCA and MRS plates for the first batch of fermentation and preliminary tests as shown in Tables 3 to 7 were conducted. From these tests, the composition of bacteria was obtained.

A mixed group of micro-organisms were present on the seeds before dehulling and boiling . These included , Gram-positive,catalase positive or weakly positive rods which metabolised glucose fermentatively in H&L medium and tentatively identified as *Lactobacillus* spp. These consisted of more than 50% of the microflora on the seeds. Also present were Gram-positive , catalase-positive, oxidase negative rods with or without spores (*Bacillus* spp?) less than 25 % of the total number of isolates. The rest of the isolates did not grow when subcultured.

On the millet flour the following groups of bacteria were found: on the PCA plates were Gram-positive, catalase-positive ,oxidase - negative/positive rods with spores (*Bacillus* spp.) 20%; Gram- positive, catalase- negative oxidase negative rods (Lactobacillus ?) 40%; Gram-negative, catalase-positive, oxidase negative actively motile yellow pigmented rods < 20% . On the MRS the flora consisted of 16% yeasts,

20% Gram-positive cocci in pairs and tetrads, catalase-negative oxidase-negative with colony morphology like *Pediococci ;* 43% Gram-positive, catalase-negative oxidase negative rods in chains or pairs; 10% catalase -weakly positive, oxidase negative rods.

The composition of bacteria on the dehulled seeds after second boiling was, 58% *Bacillus* spp which were Gram-positive catalase positive oxidase positive rods with spores, 17% Gram-positive catalase positive oxidase -negative cocci yellow and red colonies; 25% did not grow when subcultured in Nutrient broth. On the MRS plates 22 out of 22 isolates subcultured were all Gram-positive catalase -negative oxidase negative short rods in pairs singles with few short chains 17 of which metabolised glucose fermentatively in H&L medium (*Lactobacillus* spp.) It should be noted that the sample was taken after the seeds have been drained in baskets and not from the pots immediately after boiling.

After millet flour was added to the dehulled seeds the composition of the bacteria was 69% Gram-positive catalase- positive oxidase positive rods with spores (*Bacillus* spp.); 14% Gram-positive catalase positive oxidase positive rods with no visible spores and 14% of the isolates did not grow on Nutrient agar. On the MRS plates the composition was Gram-positive catalase -negative oxidase negative rods 52%; Gram - positive catalase-negative, oxidase negative coccobacilli in pairs(*Leuconostoc*?) 12%; Gram-positive catalase -negative oxidase negative cocci in pairs and tetrads (*Pediococcus*?) 8% and the rest were not identified.

The composition of bacteria after 24 h fermentation was not determined due to many problems encountered with contaminations and the breakdown of the autoclave.

The composition of bacteria after 48 h fermentation was made up of mainly *Bacillus* spp. Of the 13 isolates examined from the PCA plates 12 were Gram positive long thin rods in pairs with or without spores , catalase positive oxidase negative or positive with colony morphology like *Bacillus spp.* Only one isolate was Gram positive cocci.

On the final product after drying, milling and packaging the flora consisted of Gram positive coccobacilli in pairs and chains , catalase negative oxidase negative possibly *Leuconostoc spp* and *Bacillus* spp.

Isolates of *Bacillus* species preserved on slants and stored at 4^{0}C

At the time of my departure, only isolates from the dehulled seeds and millet flour at the beginning of fermentation had been selected and preserved on Nutrient Agar slants and kept at 4 ^{0}C. These included 18 *Bacillus* spp. 14 of which were from the cooked seeds plus millet flour and 4 from the cooked seeds listed below:

Bacillus spp. from dehulled seeds after second cooking plus millet flour (B12b)

B12b1, B12b2, B12b3, B12b4, B12b6, B12b9, B12b13, B12b14, B12b15, B12b16, B12b17, B12b18, B12b24, B12b29.

Bacillus spp. from dehulled seeds after second cooking (B12a)

B12a10, B12a20, B12a21 and B12a22.

Moulds

On the seeds of *Parkia biglobosa* the dominating mould species were *Aspergillus*, *Penicillium* , *Mucor* and a few unidentified yeasts. Moulds isolated from the millet flour were mainly *Fusarium* spp. and *Aspergillus* spp. No moulds were detected after dehulling and boiling of the seeds and during fermentation. But after drying the fermented material, 1.5 x 105 cfu/g consisting of *Aspergillus* and *Penicillium* spp. and a few unidentified mould spp. Similar flora was found on the milled and packaged product but in less numbers.

pH measurements

The pH of the dehulled seeds plus millet flour at the beginning of fermentation was 4.7 -5.0. This increased sharply to 7.6 - 8.1 after 24 h fermentation after which there was a slight increase to 7.9 -8.7 at 48 h fermentation.

a_w measurements

The a_w of the dried seeds was 0.45-0.46. After boiling and dehulling of the seeds, the aw increased to 0.94 and did not seem to change much during the fermentation.

Table 1. Results of microbiological examination of Soumbala fermentation process
Batch 1 samples taken from the pilot plant from 7 th June to 23 rd June 1997.

Sample	pH	a_w	cfu/g PCA	cfu/g MRS	cfu/g MA
Raw seeds of Parkia biglobosa	5.1	0.45	1.1×10^3	3.7×10^3	6.8×10^3
Millet flour	nd	nd	5.6×10^6	5.0×10^5	5.0×10^4
Dehulled seeds after second boiling	4.7	nd	4.9×10^4	9.6×10^3	$<10^1$
Dehulled seeds + millet flour at beginning of fermentation	4.7	0.94	1.0×10^4	8.7×10^2	$<10^1$
24 h fermentation	7.63	0.96	$>2.8 \times 10^8$	$>10^5$	$<10^1$
48 h fermentation	7.9	0.94	2.5×10^8	$>7.5 \times 10^7$	$<10^1$
Dried product before milling	7.4	0.42	$>10^7$	6.4×10^5	1.5×10^5
Milled product	7.4	0.41	3.5×10^8	9.1×10^5	1.0×10^3

Table 2. Results of microbiological examination of Soumbala fermentation process
Batch 2 samples taken from the pilot plant from 24 th June to 30 th June 1997.

Sample	pH	a_w	cfu/g PCA	cfu/g MRS	cfu/g MA
Raw seeds of Parkia biglobosa	5.4	0.46	$<10^5$	1.7×10^6	nd
Millet flour	nd	nd	nd	nd	nd
Dehulled seeds after second boiling	5.0	0.94	5.3×10^3	7.5×10^2	$<10^1$
Dehulled seeds + millet flour at beginning of fermentation	5.0	0.94	5.8×10^4	1.1×10^2	$<10^1$
24 h fermentation	8.1	0.92	4.1×10^8	5.0×10^6	$<10^1$
48 h fermentation	8.7	0.84	8.0×10^9	9.6×10^8	$<10^1$
Dried product before milling	6.8	0.43	$6.4 \times 10^{8*}$	*	$5.0 \times 10^{1*}$
Milled product	6.8	0.41	$4.1 \times 10^{7*}$	*	$8.0 \times 10^{2*}$

* Plates were contaminated

Implementation of Good Laboratory Practice (GLP)

An attempt was made to implement GLP and instructions given to enable full implementation when some requirements which were identified during the visit have been provided. These guidelines are attached.

General Observations and Recommendations

The general cleanliness and layout of the new laboratory is good. Also the new inoculation room is a good addition to the laboratory facilities. A few requirements will be needed to augment the present facilities and these will be mentioned in the following paragraphs

Staff

The present personnel in the microbiology section consist of one trained technician and two auxiliary staff. One has no educational background. The other has some basic education but no technical training and acts as a link between local Soumbala industry and the laboratory.

There is only one scientist (microbiologist) in the person of Dr. Diawara who is also the Director of the Department.

Recommendations

1. For continuity of the project work and efficient functioning of the laboratory, it is recommended that another trained technician be employed.

2. A junior microbiologist is also needed to assist the Director in the research and over-all supervision of microbiological work.

3. The staff without any formal educational background be trained fully to handle cleaning and washing of glassware. He should be properly supervised in carrying out this work.

4. The third staff who is the link between the local processors of soumbala should also be trained to help with sterilization of glassware , preparation and autoclaving of media and other simple technical work.

5. **Additional Equipment and materials needed**

The following is a list of items proposed for purchase:

- Small autoclave for melting media
-Another incubator
-Drying cabinet for glass ware
-pH meter
-Glassware / conical flasks/ blue caps of 2 l volumes; petri dishes
-dispensette
-Filter holders and O.2µ filter pads
- Autoclave tape
- Hot air oven tape
-Thermometers for hot air oven with temperature up to 200^0 C, partial immersion thermometer for incubators, low temperature thermometers for refrigerators and deep freezers.

Guidelines to help implementation of Good Laboratory Practice

Cleaning

Laboratory

* The floor should be wet -mopped and disinfected daily to prevent dust build up.

* All shelves and surfaces should be cleaned with a moistened rag daily.

* Records of cleaning should be kept in cleaning book.

* Inspection of the lab to be conducted daily to see if the level of cleaning is good.

Inoculation room

The table tops should be cleaned with 65% alcohol and not with absolute alcohol.

Formaldehyde solution should be placed in the room to fumigate it on a monthly basis or As may be found necessary.

- It is also recommended that a wash basin should be placed here.
- A hand towel should also be provided and changed on a daily basis
- It is mandatory to wear laboratory coat before entering this laboratory

Equipment

Incubators

* Once a month the interior of all the incubators should be cleaned with a mild detergent rinsed and dried with soft cloth and recorded in the cleaning book.

Water baths

* The exterior should be cleaned with mild detergent and rinsed and cleaned with a soft cloth. These should always be filled with distilled water.

Refrigerators

* The exterior should be cleaned with a damp cloth monthly. They should be defrosted every 3 months and interior cleaned.

Autoclave

* Autoclave should be cleaned weekly. The strainer should be removed to collect all lint and sediment. This should be recorded in the cleaning book.

Hot air ovens

* On a monthly basis the interior of the hot oven should be cleaned with a mild detergent solution, rinsed and dried and record of cleaning kept in the cleaning book.

pH meters

The casing of the pH meter must be kept clean. Electrodes should always be rinsed when being transferred between standard and sample solutions. After thorough rinsing blot with a soft tissue paper. Before

each use, the pH meter must be standardised with one or 2 buffers pH 4 and pH 7.

Glassware

* Glass ware that have growth in them should be autoclaved before washing with liquid detergent,

* Petri dishes should be air-dried before sterilisation

Equipment Performance Tests

* Performance tests should be carried out for all major equipment in the microbiology laboratory and documented.

Incubators

* Measurements of temperature first thing in the morning before analytical work begins. Adjustments should be made where necessary. Records of both corrected temperatures should be recorded in a book, date and initials of staff also recorded.

Refrigerators and freezers

* All refrigerators should be maintained at 4°C. On a daily basis ,reading of temperature should be made and corrected if found necessary. These should be recorded in the book with date and initials of staff.

Autoclave

* The autoclave must be able to maintain temperature of 121°C under a pressure of 1 bar (15 psi); it must be able to reach this temperature within 30 minutes. A service specialist should be called to check and service the autoclave to attain a temperature of 121°C. Thermocouple reading at various points within the chamber should be done annually by the service specialist and recorded in the autoclave book.

* Autoclave tapes be used with each load. Each load should be entered in the autoclave book stating temperature attained and time used , date and initials.

* Procedures for running the autoclave should be written up and made available for reference.

Hot air Ovens

* Hot air oven indicator tape is recommended.

* Records should be kept to indicate date, load, duration of sterilisation, temperature of operation and initials,
* Temperature should be measured with a special high temperature thermometer capable of measuring temperature up to 200°C. Graduation should not exceed 1°C..

* Glass ware should be sterilised at 160°C for 2 hours or 180°C for 45 minutes.

* The procedure for running the Hot air oven should be written up and made available for reference.

pH meters

* Before each use, the pH meter must be standardised with one or 2 buffers pH 4 and pH 7. Electrodes should always be rinsed when being transferred between standard and sample solutions. After thorough rinsing blot with a soft tissue paper. Every pH measurement must be recorded in the pH book, stating the sample pH, date and initialled

Manuals

* All manuals for all equipment should be kept in a file and made available for reference whenever necessary. Manuals of equipment supplied by DANIDA are in English. It is recommended that French translations be made as soon as possible.

Media

* Date of receipt of media should be indicated on the container and date opened should also be indicated.
* Expired media should not be used .

Preparation and use of media

* Media should be prepared in bulk in a flask of at least twice the volume the amount being prepared.

* The dehydrated medium should be allowed to completely dissolve by stirring and gentle heating before dispensing.
* pH of media should be measured before autoclaving and if necessary adjusted with 1 N HCL or 1 N NaOH before autoclaving. The pH after autoclaving should also be measured . All measurements to be entered in the media preparation book with dates and initials.
* Autoclaved media to be used for pour plates should be allowed to cool to 44- 46^{0} C before use but should not be left for more than 3 hours at this temperature.

Audits

Periodic audits should be conducted to determine whether all operations are being followed and corrective action taken.