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STARCH HYDROLYSIS BY AMYLOLYTIC LACTOBACILLUS AND UTILIZATION IN SOLID FERMENTATION OF CASSAVA

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INTRODUCTION

Cassava cultivation is increasing in tropical countries of Africa, Asia or America, because it accepts hard climatics conditions and poor soil fertility. In Africa almost all the 45 million metric tons produced each year are consumed as human food and represent the main energetic food resource for 300 million people; in some countries Cassava can represent more than 75% of energetic need.

On the other hand, Cassava have 3 bad caracteristics:
- its toxicity in relation to cyanogenic glucosides in roots
- poor nutritional quality in relation to the very low content in proteins
- poor conservation of fresh root.

Traditional fermentation practices were designed by people to limit these 3 bad points, specially for detoxification. A large part of such traditional processing have a fermentation stage, which can use intact root or pulpe like in the case of attieke or gari in Africa. In all cases the fermentation takes 2-6 days and the product has to be processed immediately without possibility of conservation.

Many microorganisms were identified, but authors generally recognize importance of lactic bacteria. The acidic fermentation also produces caracteristics flavors of the fermented food.

In the case of gari or attieke, fermentation conditions are relatively constant, but in other cases, root fermentations processing are not well controled.

Generally, cyanhydric acid residus in Cassava food are not involucred in high intoxication, but can be implemented in the etiology of some illness wellknown in regions were people consume a lot of Cassava. Thus it is important to study such traditional processing in the view of better efficiency, allowing to keep rules of quality control.

Our major research objectives on Cassava transformations are for:
- protein enrichment and nutritional value
- bacterial fermentation for detoxification of cyanhydric acid
- lactic fermentation for conservation of Cassava or fermented food.

Initially we studied on Cassava protein enrichment for animal feeding, working on solid state fermentation process. One point of special interest was to design technics for conservation of Cassava roots, and for that we considered the alternative of silage technique using lactic acid bacteria.

Here we are speaking of Cassava lactie fermentation, with special attention to amylolytic lactic bacteria.

At first, we isolated amylolytic lactic bacteria and studied in details one strain of Lactobacillus for its physiological properties; then we used this bacteria for inoculation of silage in comparison with natural "silage" of Cassava.

LACTIC ACID BACTERIA

Lactic bacteria are mainly Lactobacillus, Streptococcus, Leuconostoc y Pediococcus. All are Gram+, and cannot produced spores. Their natural media inclued vegetal material, rumen , milk and generally very rich in protein and vitamin media.

Their energetic metabolism is exclusively fermentative. They don't have cytochrom, and cannot produced energy from respiratory system. They do not synthetize catalase, but lactic bacteria are not strictly anaerobe bacteria, because they can eliminate peroxydes using NADH peroxydase in the same way than catalase (Gasser, 1975). All these caracteristics allow rapid identification of lactic bacteria by the Whittenbury method (1964).

Lactic bacteria are divided in two groups following the product of glucose catabolism:
- Homolactic bacteria, when 1 Glucose ---> 2 Lactic Acid
- Heterolactic bacteria when 1 Glucose ---> 1 CO2 + 1 lactic acid
  + 1 (acetic acic or ethanol)

Starch hydrolysis by lactic bacteria was studied only for Streptococcus, and specially for Streptococcus bovis from rumen (SEELEY & DAIN, 1960). This amylolytic capacity is a caracter exceptional mainly for Lactobacillus (Buchanan & Gibbon, 1974).

Some reports described succesfull attempts for getting silages from Cassava roots (SERRES & TILLON, 1972) or from banana refuse (LE DIVIDICH et al., 1976). But no microbial studies exists on such possibility of Cassava conservation techniques.

ISOLATION AND SELECTION OF AN AMYLOLYTIC LACTOBACILLUS

Lactic bacteria were isolated from fresh Cassava grounded in pulp with 2.5% NaCl, in bottles with anaerobic conditions. From juce of the silage, isolation were performed on ROGOSA medium; Petri dishes were placed in anaerobic jar at room temperature or 40xC.

By this method, we isolated 15 strains of lactic bacteria, of which 2 were Leuconostoc, 5 Streptococcus and 8 Lactobacillus.

We studied specially Lactobacillus beacause they are more acid tolerant and they do not have pathogen risk. Among the 8 strains isolated, 5 could grow on starch medium, but for only 2 strains it was possible to identify starch hydrolysis area by iode test in petri dish. One Lactobacillus with a special large hydrolysis area (6 mm) was selected for further studies: LA41

Optimum temperature is 40xC and optimum pH of the medium is 5.5. This strain do not produce gas from glucose. Lactic acid is the main metabolite indicating that this strain is homofermentative. All the tests indicate that the strain is close to Lactobacillus acidophilus with exception for starch hydrolysis capacity and for not growth on mannose.

PHYSIOLOGY OF THE AMYLOLYTIC LACTOBACILLUS

In a first step we compared growth kinetics in Biolafitte fermentor without aeration but with pH control. Measurement of ammonium utilization for pH adjustment served to apreciate lactate production, considering that all the acidification was due to lactic acid synthesis. The medium composition was the following (in g/l): Glucose or Cassava flour, 10 ; Yeast extract, 2.5; Trypticase, 2.5; (NH4)2 SO4, 3; K2HPO4, 2.5; NaCl, 2.5; pH=5.5

- Growth on glucose

Results are reported on the TABLE I. We can consider 3 stages:
- between 0 and 4 hours, exponential growth of the Lactobacillus
- 4-5 hours, slowing phasis
- 5-8 hours, Biomass in "resting cells", constant production of lactic acid, untill all the glucose was consumed.

The doubling time for biomass was aproximately 1 hour during the exponential period.

The transformation ratio of glucose into lactate was near 93%

- Growth on Cassava starch

From Cassava medium, kinetics is for the essential comparable with glucose. But, like it can be observed from results on TABLE II, there is a Diauxie phenomena, which is caused for initial consumption of reducing sugar present in cassava flour, then after an adaptation and amylase synthesis, which allowed growth from starch.

On starch, the growth rate was 1.1 - 1.15 hour. The growth stopped when reducing sugar were accumulating, what indicated that growth was limitated by growth factor and not by starch hydrolysis rate.

In the same way than for glucose all consumed sugars (91%) were transformed into lactate; The starch hydrolysis of Cassava was 84%; and the global tarnsformation of starch into lactate was 75-77%.

- Energetic uncoupling

In our media, the Lactobacillus strain exhibited a rapid uncoupling between growth and lactic acid synthesis. TURNER (1975) and BREHÈNY (1975) observed such uncoupling with Streptococcus, which produced lactic acid during the resting cells phasis.

We confirmed this uncoupling by addition of chloramphenicol during the initial growing phasis. Immediately the growth stopped, but the lactic acid synthesis continued linearly, what is the proof that growth and acid production are perfectly uncoupled.

- Synthesis of amylase

The amylase activity of liquid medium during fermentation of starch Cassava indicated that the synthesis of the amylase was performed not only during the growing phasis, but also during the stationary step at a linear rate. Moreover, we observed a correlation between amylase synthesis rate and concentration of reducing sugar in the medium.

- Properties of amylase

Above results indicate that the amylase is not constitutive, but inducible. On the other hand, the synthesis of the amylase was submited to catabolite repression of glucose. That was confirmed by addition of glucose during fermentation of cassava starch; immediately the amylase synthesis stopped, amylase sythesis started again only when all the reducing sugar was evolved.

The more interesting is the capacity of the Lactobacillus to synthetize amylase during the resting cells step, because in poor media like crude cassava, it is possible to perform some starch hydrolysis and acidification.

Chromatographic analysis indicated that maltose represented 95% of sugars initially. Thus the amylase of this Lactobacillus is a beta-amylase.
We determined optimum pH activity between pH=5.5 and pH=4. Optimum temperature was between 60 and 40xC.

- Transformation of starch into lactate

Lactic bacteria require high content in growth factors, and we cannot observed growth of Lactobacillus on poor cassava medium without addition of yeast extracts or trypticase. But lactic bacteria can transform starch into lactic acid without growth. We confirm this possibility by inoculation with a biomass of Lactobacillus in a simple medium containing 20 g/l of cassava flour, 3 g of (NH4)2SO4, 2.5 g of K2HPO4 and 2.5 g of NaCl. In such a medium we observed no growth of the Lactobacillus, but a linear production of lactate with a slope depending of the biomass concentration.

The same experiment indicated that the production stopped when the concentration of lactic acid was 13 g/l, which is the toxic level for that strain. It indicated also the possibility of starch transformation without bacterial growth in poor media.

CASSAVA SILAGE CONSERVATION

The amylolytic Lactobacillus is capable of transformation of starch into lactic acid with a 75% ratio. It was interesting to test the potentiality of inoculation of cassava pulp with this strain for the stabilization of the material thanks to lactic acidification.

For that we compared natural silage of Cassava with inoculated silage by the strain of amylolytic Lactobacillus. The results are reported on TABLE III and IV.

For inoculated silages, pH decreasing was rapid, and after 2 days, pH was about 4.3 and 75% of lactic acid was produced. For natural silages, pH decrease was sensibly slower and pH stabilization between 4.9-5.0. The lactic acid content was about 10-12 g/Kg in natural silages and the double (20-24 g/Kg) in inoculated silage.

The inoculation of Cassava with the Lactobacillus inhibited development of others bacteria; for that the gas evolvement was important in natural silage and zero in inoculated silage.

The inoculation produced a better acidification and stabilization of the Cassava pulp, in comparison with natural fermentation.

- Influence of starch concentration

In the last experiment we studied effect of starch concentration on the acidification and lactic acid production. The results reported on TABLE V indicate a maximum acid production after 14 - 21 days of silage. More concentrated was the medium (untill 500 g/l) more lactic acid was synthetized. The maximum concentration was 22.4 g/l of lactic acid and pH was stabilized around pH=3.4. However, we observed decreasing in lactic acid content indicating some lactic degradation, which could be a problem for a very long period of conservation (more than 2 or 3 months) however the pH remained very acidic (pH = 3.4) during the same period.

PROTEIN ENRICHMENT FROM CASSAVA SILAGES

Finally we studied the possibility of utilization of cassava silages for protein enrichment following solid fermentation techniques (RAIMBAULT et al., 1980) through mould inoculation. Results are reported on TABLE VI. We can observe :
- rapid degradation and utilization of lactic acid by Aspergillus in both cases
- better protein enrichment with flour obtained from inoculated silage (2.1 versus 8.1 % on the dryed matter basis).

CONCLUSIONS

The results indicate :

- Amylolytic Lactobacillus strains can be isolated from Cassava or traditional fermented food

- Homolactic Lactobacillus can be utilized for Cassava conservation for long period by rapid acidification and stabilization of starch degradation

- Uncoupling of growth and lactic acid production in addition to beta-amylase synthesis by a strain of homofermentative Lactobacillus make possible the conservation of crude pulp of cassava.

- Cassava silage can be used for further processing, including protein enrichment and mould bioconversions.

But more researches are necessary about lactic acid bacteria and specially for amylolytic bacteria, because:

- Lactic bacteria, and specially Lactobacilli present the caracteristic to be not very stable.

- Amylase capacity of Lactobacilli is not very efficient in comparison with strains like Bacillus subtilis and for that we focus our interest on techniques for improvement of such hydrolysis capacity by mutation techniques or by genetic transformation techniques

- It would be interesting to consider mixed starter containing:
  Flavoring strains
  Amylolytic Lactobacillus
  Laminarase producers

For the moment we are working on molecular genetics of Lactic acid bacteria with the objective to get efficient amylolytic Lactobacillus homofermentative. For that we planned to study
- Plasmid identification of amylase; radiation of plasmid
- Molecular genetic techniques to transform a strain of wellknown Lactobacillus with amylolytic capacity using Bacillus subtilis

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TIME (H)	GLUCOSE (G/L)	LACTATE (G/L)	O.D
0,00	0,00	0,00	0,05
1,00	0,10	0,05	0,06
2,00	0,33	0,31	0,13
3,00	0,80	0,70	0,29
4,50	3,40	1,80	0,68
5,00	4,50	3,60	0,75
5,50	5,30	4,20	0,80
6,00	6,10	5,00	0,85
7,00	7,60	6,60	0,89
8,00	9,00	8,10	0,90
9,00	9,90	9,10	0,90
10,00	10,00	9,30	0,87
11,00	10,00	9,30	0,85
12,00	10,00	9,30	0,85

TABLE I.- Growth of *Lactobacillus* on Glucose medium (10 G/L).

TIME (H)	GLUCOSE (G/L)	LACTATE (G/L)	O.D
0,00	0,00	0,00	0,35
1,00	0,10	0,10	0,37
2,00	0,38	0,31	0,42
3,00	0,70	0,73	0,50
4,00	1,05	1,05	0,58
5,00	1,53	1,40	0,66
6,00	2,24	1,90	0,81
7,00	3,65	2,63	0,94
8,00	5,37	4,20	1,00
9,00	6,97	6,00	1,10
10,00	8,17	7,37	0,99
11,00	8,41	7,70	1,00
12,00	8,42	7,70	1,10

TABLE II.- Growth of *Lactobacillus* on Cassava flour medium (10 G/L).

TIME (day)	Without INOCULATION	INOCULATED
0,00	0,51	0,51
0,25	0,78	2,57
0,50	1,23	6,58
1,00	3,52	11,92
2,50	7,75	16,51
7,00	11,00	20,65
12,50	12,37	21,42

TABLE III.- Lactate production in silage with or without inoculation by *Lactobacillus*.

TIME (day)	Without INOCULATION	INOCULATED
0,00	6,15	6,20
0,25	6,00	6,00
0,50	5,80	5,20
1,00	5,35	4,60
2,50	5,15	3,90
7,00	5,00	3,55
12,50	4,90	3,50

TABLE IV.- Changes of pH in silage with or without inoculation by *Lactobacillus*.

	CASSAVA FLOUR CONCENTRATION				
	100 g/l	200 g/l	300 g/l	400 g/l	500 g/l
TIME (H)	ac. Lact.	ac. Lact.	ac. Lact.	ac. Lact.	ac. Lact.
24	4,56	7,11	8,47	8,07	4,60
66	4,45	8,43	10,92	13,33	13,98
114	6,67	10,18	9,44	11,68	16,02
330	7,36	10,75	12,56	13,77	22,38
500	8,46	10,12	13,74	12,62	14,27
715	7,11	8,80	12,63	8,54	18,32
860	N.D.	N.D.	10,51	N.D.	15,96

TABLE V.- Effect of Cassava flour concentration on lactic acid concentration in silage inoculated with *Lactobacillus*.

	NATURAL SILAGE			INOCULATED SILAGE		
TIME (Hours)	Lactic acid %	Sugar %	Protein %	Lactic acid %	Sugar %	Protein %
0	1,60	77,00	1,90	4,20	77,20	1,90
15	1,60	78,20	2,20	4,20	77,50	2,20
22	1,50	78,20	3,20	3,20	75,60	4,70
27	1,00	70,00	3,80	1,70	69,80	6,30
30	0,80	74,60	5,00	1,00	57,90	8,70
40	0,40	57,30	8,10	0,20	39,80	12,10

TABLE VI.- Protein enrichment by solid state cultivation of *Aspergillus* using Cassava flours from natural and inoculated silages.