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Full Length Research Paper

Cassava solid-state fermentation with a starter culture of *Lactobacillus plantarum* and *Rhyzopus oryzae* for cellulase production

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A mixture of two microorganisms was used as starter culture, with the aims of producing cellulose from fermented cassava roots. Cellulase yields of 430 IU/g were recorded in solid-state fermentation of cassava starch with mixed culture of *Lactobacillus plantarum* and *Rhyzopus oryzae,* isolated from cassava dried chips. High cellulase activity (8.6 IU/ml) per unit volume of enzyme broth and high yields of cellulases were obtained and attributed to the growth of both strains on cellulose fraction of cassava roots. In Solid State Fermentation with Starter mix-culture (SSFS), fermentation retting was completed in two days, and significant softening was noticed. No significant softening was evidence in Solid State Sterile Fermentation (SSSF) and Liquid state fermentation (LSF).

Keywords: Cassava, cellulases, *Lactobacillus plantarum, Rhyzopus oryzae*, solid state fermentation, liquid state fermentation.

INTRODUCTION

Cassava (*Manihot esculenta Crantz*) is a tropical root crop, which is a staple food and a major source of calories for over 800 million peoples in developing countries (Cock, 1985). However, the roots contain cyanogenic glucosides, linamarin and lautaustralin, which are responsible for chronic toxicity associated with the continued ingestion of poorly, processed cassava products (Howlet et al., 1990; Mlingi et al., 1992; Dimuth and Richard, 2004). Due to the perishability and high cyanogen contents in the roots, several village processing techniques have led to the development of different cassava products for human consumption straw; LSF, liquid state fermentation (Lancasterand and Coursey, 1988). This traditional processing includes, boiling, smoking, drying, and retting which, is by far the most important and widely used means of processing (Oyewole, 1992; Westby, 2002). Retting is a spontaneous fermentation of cassava in Central Africa, traditionally performed to soften the roots, degrade endogenous cyanogenic compounds and to give cassava-based foods theirs specific flavor (Kobawila et al., 2005). During this step, degradation of plant cell occurred and led to cassava softness. Various bacteria have been found to be able to produce pectinases, some lactic acid bacteria such as *Lactobacillus plantarum* and *Leuconostoc mesenteroides* two main bacteria of cassava retting have been reported to process polygalacturonase and pectate lyase.

The present work attempts to describe solid-state-fermentation process to the cellulase production by mix starter culture of *Lactobacillus plantarum* and *Rhyzopus oryzae*, two strains of microorganisms isolated from cassava dried chips, with the scope of elaborating a bacterial and yeastmix culture starter. This could lead to development of a new source of cellulase production from cassava roots.

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Abbreviations: SSSF, solid state sterile fermentation; **SSFS,** solid state fermentation with starter mix-culture; **MRS,** de Man Rogosa Sharpe; **PDA,** potatoes dextrose agar; **WS,** wheat.

MATERIALS AND METHODS

Isolation and identification procedures

About 1 Kg of fermented cassava chips were collected and divided in 100 g parts. Each part was mixed with 900 ml of sterile 0.1% peptone water to form a solution. Serial dilutions were then made from solutions and plated. The lactic acid bacteria were isolated on de Man Rogosa Sharpe (MRS) agar as described by Sharpe et al. (1966) and Yeast in Potatoes Dextrose Agar (PDA). Plates were incubated at 30°C for 24 to 48 h. Isolates were then randomly picked at varying times from PDA and MRS plates and subcultured in 10 ml tubes, and subjected to physiological and biochemical test (Howlet et al., 1990; Mlingi et al., 1992). Identification method was based mainly on the following; microscopic and macroscopic examination, mobility, presence spores, catalase test, gram stain reaction, growth temperatures, homofermentative and heterofermentative character, thiamine requirement for growth, utilisation and fermentation of different sugars and other carbon containing substances (API 50 CH N° 5030 strip, biomerieux charbo nière, bains France). Lactobacilli *(L. plantarum)* and yeast (*R. oryzae)* species were taxonomically classified following the discriminatory schemes of Kandler and Wiess (1986), Hammes et al. (1992), and Larpent and Larpent-Gouraud, (1985).

Screening for cellulase production

Amylolitic strains of *L. plantarum* and *R. oryzae* were continuously maintained on delignifield wheat straw (WS) agar medium in Petri plates. The WS agar medium was specially designed for this purpose and the nutrients in the medium are described below. In one of the colonies, a sector showing high hydrolytic activity was noticed. The mutation occurred due to continuous subculturing on WS medium. New mutants were transferred to a WS - cassava based medium, and in five further transfers, a stable mutant were obtained. An hyper-cellulase-producing mutant of *T. reesei*, NRRL 11460 (Rut-C30), receive from J.J. Ellis, Northern Regional Research Center, Periora, III., was also used for comparison. It is referred to below as Rut-C30.

Substrates

WS, ground to 20-mesh powder, was used as a source of cellulose. WS contains (percent dry weigth): cellulose, 40; hemicelluloses, 29.2; lignin, 13.6; protein, 3.6; and other materials, 13.6 (Harrigan and Mc Cance, 1976). Therefore, Cheat straw is composed of 70% insoluble carbohydrates suitable for the growth of microorganisms and for cellulase production.

Cassava roots were prepared by a chemical-thermomechanical process as described by Law et al. (1983) and the roots thus prepared were called chemithermomechanical cassava roots (CTMCR). During this process, the wood was pulverised to fine fibres which still retained most of the hemicelluloses and lignin. The chemical composition of CTMCR was as follows (% dry weight): cellulose 35-46; hemicelluloses, 10 to 15; and lignin, 5 to 8.

Pre-treatment

Powdered substrate (5 g; 20 meshes) was dispensed into each Erlenmeyer flask of 250 ml capacity. Each substrate was treated with NaOH (4% wt/wt) with 33.3% moisture at 121°C (W S for 0.5 h and CTMCR for 1 h). The treated substrates were not washed. All of the solubilized hemicelluloses and lignin were retained in the fermentation medium. After the addition of nutrients, the pH was adjusted to 5.8 with H_2SO_4. Water soluble was obtained by suspending treated WS in water (1/10) and filtering through four layers of cheese cloth. The wheat straw soluble contained 2.5% solids (hemicelluloses, lignin, and other cell solubles).

Nutrients

Nutrients described by Mandel and Weber (1969) for cellulase production were supplied in concentrated for, but proteose peptone was replaced with Yeast extract (Difco Laboratories, Detroi, Mich). The quantity of nutrients required for each substrate was determined at the rate of their carbohydrates content. The required amount of concentrated nutrient salt solution (5 ml for WS and 5.7 ml for CTMCR) was added to 5 g of substrate. The concentrated salt solution contained the following, dissolved in 200 ml of water: KH_2PO_4, 28 g; $(NH_4)SO_4$, 19.6 g; Urea, 4.2 g; $MgSO_4$ $7H_2O$, 4.2 g; $CoCl_2$, 4.2 g; $FeSO_4$ $7H_2O$, 70 mg; $MnSO_4$ $7H_2O$, 21.84 mg; $ZnSO_4$ $7H_2O$, 19.6 mg; $CaCl_2$ 28 mg; and Yeast extract, 7 g. As referred to below, a full concentration of nutrient means the complete required quantity of nutrients as mentioned above, whereas a one half concentration is one half of that quantity. All of the flasks were autoclaved at 121°C for 20 min, after the nutrient sa lt solution was mixed with the substrate.

Moisture

The moisture content of the substrates after pre-treatment and the addition of nutrients and inoculum was 80% (wt/wt) in solid state fermentation (SSF). Sterilizer was added for liquid state fermentation (LSF), to obtain the desired concentration of substrate in the fermentation medium.

Starter preparation

Inocula of mutants *L. plantarum* and *R. oryzae*, were produced on the modified medium as described above but containing 1.5% glucose, with the nutrient salt solution diluted accordingly. For inoculation of each flask containing 5 g of substrate, 5 ml of 48 h old mix-culture (2.5 ml of each strains) was used. The inoculum was spread on the surface of the substrate.

Cultures conditions

All of the SSF cultures were incubated at 30°C in a h umidified incubator (about 80% relative humidity), whereas the LSF cultures were incubated at the same temperature on a shaker at 150 rpm.

Extraction of cellulase system

The culture of SSF from each flask (originally 5 g of substrate) was properly mixed with more water to bring the final weight of the mixture (mycelium and bacteria plus unutilised lignin, cellulose, and hemicelluloses) to 100 g. Tween 80 was added at a rate of 0.1%. The mixture was shaken for 0.5 h and centrifuged. The supernatant was used for enzyme determination. It was estimated that about 7 to 10% cellulases remained adsorbed on the residues (mycelium and unutilised cellulose, hemicelluloses, and lignin) when the residues were suspended in water and Tween 80 as before and and the supernatant was tested for cellulase titre.

Biochemichal analysis

Cellulase titre was calculated in international units of enzyme

activity (glucose released per min) on filter paper for 60 min, by the method of Ryu and mandels (1980). β-Glucosidase titre was measured in international units of glucose released per min with 1% salicilin solution for 30 min. Xylanase titre was measured in international units of Xylose released per min with 1% xylan for 10 min (Ishaque and Kluepfel (1981). The cellulase yield per gram of cellulose was calculated by dividing total international units of cellulase titre in 100ml of enzyme broth by grams of cellulose present in the substrate supplied in flask.

Sugars were estimated by using a Beckman 344 gradient high-pressure liquid chromatograph with an Altex 156 refractive index detector and a Spherogel 7.5% carbohydrate column with a flow rate of 0.5 ml/min in the mobile phase of water at 80℃. The sugar samples were appropriately diluted before injection.

RESULTS AND DISCUSSION

Cellulase production in LSF and the effect of different concentration of WS medium

The highest cellulase titre (1.65 IU/ml) and cellulase yield (412 IU/g of cellulose) in LSF were obtained with mix-culture starter in 1% WS (0.4 %cellulose) slurry after 7 days (Table 1). When the concentration of WS was increased to 5% (2% cellulose), the enzyme production time was increased from 7 to 11 days. The enzyme activity increased to 6.0 IU/ml, but the cellulase yield dropped to 300 IU/g of cellulase (Table 1). The drop in cellulase yields might have been due to poor mass transfer in the thick slurry of 5% WS.

Cellulase production in SSF

On WS

SSF was carried out with a full concentration of nutrients in one set of experiments and with a one-half concentration in another set to evaluate the effect of different concentrations of salts in the medium, since some microorganisms are unable to grow in the high osmotic pressure of the medium. Starter culture (mix-micro-organisms) was quite tolerant to the high concentrations of the nutrients, as indicated in Table 1. It produced the highest enzyme titre (8.6 IU/ml) and cellulase yield (430 IU/g of cellulose) in SSF on a full concentration of nutrients, after 22 days. The highest cellulase yield, 412 IU/g of cellulose, obtained in LSF, was because that medium contained the lowest cellulose concentration (0.4%), as discussed earlier. However, in the present study, a cellulase yield of 275 to 300 IU/g of cellulose in LSF on 2% cellulose concentrations was considered for comparisons between LSF and SSF. When the nutrients were supplied in a one–half concentration, the cellulase titre dropped to 6.7 IU/ml, cellulase yield dropped to 335 IU/g of cellulose, and there was no increase in enzymes Yields after an incubation of more than 14 days. But when the cultures were stirred after 11 days of growth and further incubated for 11 days without any stirring (total of 22 days of incubation), the celulase titre decreased somewhat for the full concentration of nutrient, whereas it increased considerably (8.0 IU/ml) for a one half concentration of nutrients. This indicated that half of the quantity of required nutrients was sufficient to get an optimum cellulase titre as well as an optimum cellulase yield. This finding could contribute to a reduction in the cost of enzyme production.

On WS and CTMCR

Cellulase production by starter (mix culture) was compared with that of a strain of Rut-C30, a hyper cellulase-producing mutant of *T. Reesei,* on two different lignocellulosic substrate, WS and CTMCR. Starter produced its highest cellulase titre (over 8 IU/ml) and yields (over 400 IU/g of cellulose) on treated WS as compared with that of Rut6C30, a 6.2 IU/ml cellulase titre and a yield of 310 IU/g of cellulose. Cellulase production by both of starters (Mix culture of L.plantarum and R. oryzae,) and Rut-C30 hyper cellulase-producing mutant of T. Reesei decreased considerably on untreated WS (Table 2). The Rut-C30 hyper cellulase-producing mutant of T. Reesei, failed to grow in number of flask containing WS or CTMCR. This finding indicated that this mutant was not well adapted to such conditions of SSF.

The mild alkali treatment of CTMCR did not affect enzyme production by starter (Table 3). Because the highest cellulase titre and Yield obtained on treated CTMRC after 20 days in SSF were almost comparable to those obtained on untreated CTMCR it appeared to be a good substrate for cellulase production event without any further treatment.

The cellulase titre and Yield on CTMRC with the mix starter (Mix culture of L. plantarum and R. oryzae) and Rut-C30 hyper cellulase-producing mutant of T. Reesei (Table 3) and on treated WS (Table 2) with starter mix culture were higher than that of mutant Rut-30. The titre and yield of cellulase obtained with mix starter culture were also higher than those obtained by other workers who grew various mutants of T. reesei or of L. plantarum and R. oryzae separately on pure cellulose in LSF (Ryu and Mandels, 1980; Gallo et al., 1978; Ampe et al., 1995). The result clearly indicated that the new approach of retaining the hemicelluloses and lignin of the alkali-pre-treated lignocelluloses (WS, CTMCR) in SSF increased significantly the cellulase titre per unit volume and the cellulase yield per unit of cellulose.

Composition of the cellulase system

The cellulase system produced in SSF contained the following enzymatic activities (international unit per milli-litre): cellulase, 8.6; β-glucosidase, 10.6; and xylanase, 270. The xylanase titre was quite variable (between 190 and 480 IU/ml); however, the ratio of cellulases and

Table 1. Cellulase production on WS by mixed starter culture.

Type of fermentation (nutrient concentration)	Concentration of WS (%[wt/wt])	Concentration of cellulose (%[wt/wt])	Time of incubation (days)	Cellulase titre (IU/ml)	Cellulase yield (IU/g of cellulose)
LSF (Full concentration)	1	0.4	5	1.44±0.21	360±26
	1	0.4	7	1.65±0.12	412±42
	5	2.0	7	1.30±0.18	65±12
	5	2.0	11	6.00±0.23	300±35
	5	2.0	14	5.50±0.18	275±25
SSF (Full concentration)	20[a]	8[b]	11	6.00±0.22	300±45
	20[a]	8[b]	14	6.30±0.17	385±61
	20[a]	8[b]	22	8.60±0.12	430±67
	20[a]	8[b]	22[c]	7.40±0.17	370±58
SSF (Full concentration)	20	8	11	5.50±0.13	275±46
	20	8	14	6.70±0.18	335±35
	20	8	22	6.70±0.14	335±25
	20	8	22[c]	8.00±0.19	400±31

[a]5g of WS + 20g of water (no free water)= 20% solid in each flask.
[b]5 g of WS contains 2 g of cellulose= 8% cellulose in each flask.
[c]Cultures were stirred once after 11 days of incubation and were further incubated for 11 days without stirring.

Table 2. Cellulase production by Mixed Starter (*Lactobacillu .plantarum* and *Rhyzopus oryzae*) and Rut-C30, a hyper cellulase-producing mutant of *T. Reesei*.

Substrate (days)	Starter			
	Mix culture of *Lactobacillus plantarum and Rhyzopus oryzae*		Rut-C30 hyper cellulase-producing mutant of *T. Reesei*	
	Cellulase titre (IU/ml)	Cellulase Yield (IU/g of cellulose)	Cellulase titre (IU/ml)	Cellulase Yield (IU/g of cellulose)
Treated WS				
9	0.7±0.05	35±21	0.6±0.03	30±11
16	4.4±0.09	220±71	1.6±0.08	80±19
18	7.8±0.1	390±55	2.6±0.06	130±14
20	8.1±0.15	405±53	6.2±0.09	310±27
26	8.5±0.05	425±64	5.2±0.15	260±64
30	7.2±0.8	360±24	4.6±0.04	230±33
Untreated WS				
9	1.7±0.07	85±24	1.1±0.35	55±17
16	2.3±0.09	115±16	2.6±0.07	130±23
18	2.4±1.0	120±11	2.8±0.10	140±27
20	2.1±0.07	105±24	3.1±0.12	155±22
26	2.2±0.03	110±19	3.2±0.15	160±19
30	1.9±0.05	95±12	4.2±0.07	210±24
Treated CTMCR				
9	1.5±	45±	0.9±	27±7
16	4.7±	142±	5.3±	161±21
18	3.9±		4.5±	136±16
20	6.3±	191±	6.6±	200±14
26	6.0±	182±	6.5±	197±23
30	6.2±	188±	4.8±	145±31

Table 2. Contd.

Untreated CTMCR				
9	2.2±	67±	1.9±	57±12
16	5.3±	161±	4.1±	124±21
18	3.3±		4.1±	124±30
20	5.0±	151±	5.8±	176±34
26	5.6±	170±	6.0±	182±27
30	7.2±	218±	5.0±	152±19

Cellulose content of wheat straw, 40%; Cellulose content of CTMCR, 35-46%.
There was 5 g (dry weight) of each substrate in each flask. Substrates were treated with 4% NaOH (wt/wt) at 121℃ for 0.5 (WS) or 1 h (CTMCR) with a 1:2 (Solid to liquid) ratio.

Table 3. Hydrolysis of cassava roots with Starter mix culture.

	Time (h)								
Hydrolysis of cassava	**0**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
SSFS saccharification[a] (in%)	1.2±0.01	5.4±0.17	15.4±0.2	18.3±0.1	22.4±0.5	25.2±1.01	30.5±3.11	46.1±2.01	52±3.01
LSFS saccharification[a] (in%)	1.2±0.02	3.2±0.11	9.2±0.13	12±2.3	14±0.17	20±0.87	23±0.47	32±0.16	43± 0.17

[a]Sacharification = grams of reducing sugar × 0.9 × 100/ gram of substrate, SSFS= Solid State Fermentation with Starter culture, LSFS= Liquid State Fermentation with Starter culture.

β-glucosidase varied between 1:1 and 1:1.5 in various cellulase system preparations. These are enzyme activities when 5 g of WS fermented in SSF was suspended in 100 ml of water to extract the enzymes. The enzyme titre could be doubled (17.2 IU/ml) by extracting the enzyme in 50 ml of water. The composition of the cellulase system indicated that there was no need to add extra β-glucosidase or xylanase for the hydrolysis of pure cellulose or lignocellulose.

Hydrolytic potential of the cellulase system on cassava roots retting

The cellulases produced in SSF, performed to soften the roots, degraded endogenous cyanogenic compounds by saccharification, β-glucosidase degradation (detoxicaion). In order to determine the origin (vegetable, or starter mixed culture) of cell wall degradation and cassava detoxication, two fermentations were performed, a SSFS retting with mix SSSF. For both fermentations, 5 kg of peeled roots cut into cubes (1 cm^3) were used in 7 l bioreactors (SET 004M, Setric, Toulouse, France). Temperatures was 30℃ and. For inoculation of bioreact or, 15 ml of 48 h old mix-culture (2.5 ml of each strain) was spread on the surface of the roots. For SSSF, cassava roots cubes were sterilise with 0.1% $HgCl_2$ in ethanol according to the procedure of Okafor et al. (1984). pH and partial oxygen pressure of sterile fermentation were set on that of the control fermentation with the addition of 1N HCl and N, respectively. Penetrometry was used as An indicator of roots softening during retting. A penetrometer (PNR 10-SUR Berlin) was used to measure the consistency of the roots. Six roots sections were randomly chosen; for each section, penetrometry depth was estimated six repetitions. In SSFS fermentation, retting was completed in two days, but no significant softening was as evidence in SSSF fermentation (Figure 1). We also noticed that cellulase activity was found in cassava fresh roots (0.4 IU/ml) total cellulase activity increased in SSFS all along the process, but was stable in SSSF.

The degradation of plant wall has been shown to be due to the combined action of pectic enzymes from both plan and bacterial origin (Baterman and Millar, 1966; Ampe et al., 1995). Previous results demonstrated that cassava softening seemed to be mediated by bacteria (Baterman and Millar, 1966; Oyewole, 1990; Brauman et al., 1995). The presence of pectinesterase and lyase was only evidenced when cassava was inoculated with *Corynebacterium* spp. However, no pectolytic activity (and especially no pectinesterase) was found in fresh roots (Ampe et al., 1995). More recently, Oyewole and Odunfa (1992) have proved the presence of extracellular pectin methyl esterase during retting.

The cellulase produced in SSFS showed saccharification on cassava roots (Table 3). There was also an indication that there is a correlation between saccharification, and cassava softness after 72 h of fermentation (Figure 2). A critical analysis of literature on enzymatic hydrolysis reveal that high cellulase activity per unit volume of fermentation broth is the most important factor in obtaining sugar concentrations of 20 to 30% from hydrolysis of cellulose for ethanol production from

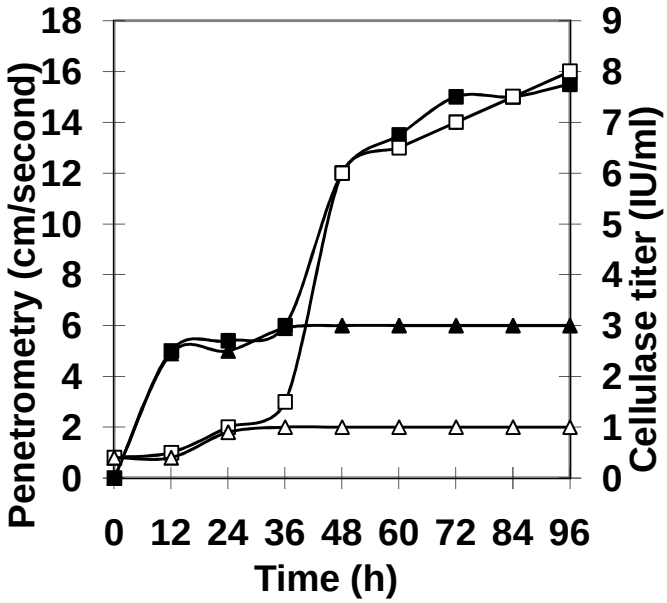

Figure 1. Cassava softness in Solid State Fermentation with Starter (■) and Solid State Sterile Fermentation (▲) and cellulase production Solid State Fermentation with Starter (□) and Solid State Sterile Fermentation (Δ).

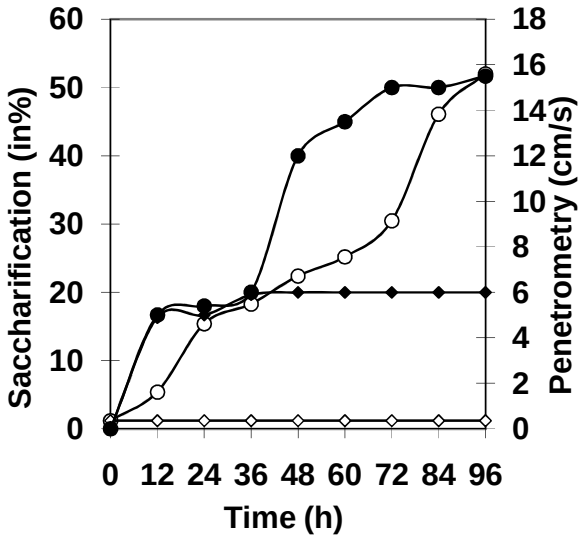

Figure 2. Cassava softness in Solid State Fermentation with Starter (●) and Solid State Sterile Fermentation (♦) and Saccharification in Solid State Fermentation with Starter (○) and Solid State Sterile Fermentation (◊).

cellulosic materials (Chalal, 1982a). It has also been confirmed that cellulase activity per unit volume increase by increasing the cellulose concentration in the medium (Nystorm and DiLuca, 1977), but it is not possible to handle more than 6% cellulose in the conventional fermentor because of rhehological problems. A maximum concentration of substrate which can be handled in the conventional fermentor is 2% for wood pulp and 6% for crystalline cellulose. Therefore, to increase the cellulose concentration to over 6%, Solid-State fermentation

seemed to be the most attractive alternative (Chalal; 1982b) and cassava roots could be an adequate substrate for this. Previous results have shown that cassava softening seems to be mediated by bacteria (Oyewole and Odunfa, 1992; Oyewole, 1990; Brauman et al., 1995). Sterile roots soaked into sterile water did not ret, whereas microorganisms of our mix starter induced wall degradation. The presence of a cellulase system (pectineesterase and lyase) was previously only revealed when cassava was inoculated with Corynebacterium spp. however, no pectolytic (and especially no pectinesterase) was found in fresh roots. More surprisingly, Okafor et al. (1984) found no cellulase activity when inoculating with Bacillus sp. whereas retting could be completed. More recently, Oyewole and Odunfa (1992) have evidenced the presence of extracellular cellulase (pectin methyl esterase) during retting.

Conclusion

This work supports the first evidence for the production of cellulase with a starter culture or mix starter culture of L. plantarum and R. oryzae cultures in different cellulose based media and their softness effect on cassava roots. The results obtained in this study suggest that cell wall degradation is initiated by the action of microbial cellulase that depolymerise pectin chains. This could lead to accelerate detoxification effect as softness has been previously shown to favour cyanogens realised in cassava roots. Those two strains (L. plantarum and R. oryzae, were selected for their exceptional capability to break down starch, their resistant to high cyanogenic glucoside levels and their ability to hydrolyse linamarin. The mix starter culture is then indicated for improvements in cassava retting process with the scope of reducing the duration of fermentation to economically viable limits, to maximize the detoxification process and improve the physical and nutritional qualities of cassava products.

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