Journal of Industrial Microbiology, 2 (1987) 123–127
Elsevier
SIM 00071

Extracellular amylase production by cassava-fermenting bacteria

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Received 3 December 1986
Revised 2 April 1987
Accepted 7 April 1987

Key words: Cassava; Starch; Amylase; Bacteria; Temperature; pH

SUMMARY

Fermentation of cassava tubers was accompanied by a gradual decrease in pH, increased amylase activity in the steep liquor, and increased microbial load and lactic acid concentration. Amylase-producing bacterial strains associated with cassava fermentation were isolated and identified as *Bacillus subtilis, Bacillus licheniformis* and *Bacillus cereus*. The pH optima for the partially purified enzymes of these organisms were 7.0, 5.5 and 7.5, whilst their temperature optima were 30, 37 and 80°C. There was no significant difference in amylase activities when starch, dextrin, amylopectin, glucose and maltose were used as growth substrates.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important staple food crop in West Africa. In Nigeria, it is processed into two main fermented products called Gari and Foufou. The microbiological and biochemical events occurring during the traditional processing of cassava have been investigated [1,4,9,10].

Fermentation of cassava has been described as a two-stage process. During the first phase, the cassava bacterium *Corynebacterium manihot* broke down the starch in cassava roots to simple sugars, and lactic and formic acids, leading to a drop in the pH of the fermenting mash [1,4]. The fall in pH also encouraged the growth of a fungus, *Geotrichum candidum*, which brought about further acidification and the characteristic aroma of the final product. Hydrogen cyanide is liberated during fermentation through the spontaneous hydrolysis of the cyanogenic glucoside of cassava at a low pH. Other workers [9,10] reported different cassava-fermenting species of bacteria and yeasts including *Leuconostoc, Lactobacillus, Alcaligenes, Candida, Pichia* and *Saccharomyces*.

Although cassava fermentation has been reported to be brought about by a mixed microbial flora, few studies have been carried out on the individual roles of the microorganisms involved. In the present study, amylase-producing strains of bacteria associated with cassava fermentation were isolated and characterized with a view to their exploitation as sources of amylolytic enzymes.

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MATERIALS AND METHODS

Isolation of microorganisms

Bacterial strains were isolated from cassava steep liquor on nutrient agar plates. Each isolate was tested for amylolytic activity on a starch agar plate that was flooded with Lugol's iodine after growth of the organism. Starch hydrolysis was indicated by clear zones around the colonies. Lactic acid bacteria were enumerated and isolated from cassava steep liquor on MRS agar plates. All isolates were characterized in accordance with the taxonomic schemes of Cowan [5] and Buchanan and Gibbons [2].

Fermentation of cassava

Freshly harvested cassava tubers were peeled, sliced and introduced into 1-litre-capacity Kilner jars containing sterile distilled water and left to ferment for 7 days, during which the total microbial load was monitored by plating out aliquots of steep liquor on nutrient agar plates. Lactic acid concentration was determined by titration of steep liquor with 0.1 M NaOH. pH values were measured by means of a pH meter (L. Pusl, Munchen, 15 model). Protein concentration was determined by the method of Lowry et al. [6] using bovine serum albumin as standard. Reducing sugar levels were monitored by the dinitrosalicylic acid reagent method of Miller [7] using glucose as standard. Amylase activities in steep liquor and subsequent culture supernatants were measured using the method of Morgan and Priest [8] and were estimated by the reducing power of starch digests. The assay tubes contained, in duplicates, 1.0% (10 mg/ml) starch solution (1.0 ml), sodium phosphate buffer (0.2 M, pH 7.0, 1.0 ml) and the enzyme solution (1.0 ml). The mixtures were incubated at 55°C for 10 min, after which the reaction was stopped by the addition of DNS reagent (3.0 ml), boiled for 10 min to allow colour development, and the absorbance was read at 620 nm in a colorimeter. One unit of amylase activity was defined as the amount of enzyme that produced 1 mg of glucose equivalent under the assay conditions.

Cultivation of amylolytic strains and enzyme preparation

Bacterial isolates were cultivated in 100-ml volumes in 250-ml Erlenmeyer flasks using a mineral salts medium which contained $NaNO_3$ 3.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, KH_2PO_4 1.0 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $CaCl_2$ 0.1 g and soluble starch 10.0 g in 1 litre of distilled water. The pH of the medium was adjusted to 7.0 before sterilization. The cultures were incubated with shaking (150 rev/min) at 30°C for 48 h. The organisms were harvested from the culture broth by centrifugation at 10000 × *g* for 25 min at 4°C. The supernatants were used as sources of extracellular amylases.

Partially purified enzymes were obtained by adding solid ammonium sulphate to the culture supernatants with constant stirring at room temperature. The precipitates were collected by centrifugation at 12000 × *g* for 20 min at 4°C, dissolved in sodium phosphate buffer (0.2 M, pH 7.0, 5.0 ml), and dialysed against two changes of distilled water at 4°C.

Effects of pH and temperature on amylase activity

The effect of pH on amylase activity was determined using buffers between pH 4 and 8. The buffers used were 0.2 M acetate buffer (pH 4.0–6.0) and 0.02 M Tris-HCl buffer (pH 6.5–8.0). The effect of temperature was also assayed between 15°C and 80°C using buffers at each organism's optimum pH.

Effect of carbon sources on amylase production

The effects of carbon sources were investigated by substituting starch with glucose, maltose, amylopectin and dextrin in the culture medium.

Paper chromatography of assay products

Products of amylolysis were assayed by paper chromatography using the solvent system butanol/acetic acid/water (80:20:20, v/v). The spots were developed with the aniline phthalate reagent.

RESULTS

Changes during cassava fermentation

The microbiological and physico-chemical changes occurring during cassava fermentation are shown in Fig. 1. The pH of cassava steep liquor decreased gradually during the period of fermentation whilst the lactic acid concentration increased correspondingly. Amylase activity and protein concentration in the steep liquor increased as the population of bacteria also increased. Reducing sugar concentration, however, increased gradually until 72 h, after which it continued to decrease.

Characteristics of amylase-producing strains

A total of eight bacterial species were isolated from cassava steep liquor, out of which only three were positive for the starch hydrolysis test. These amylolytic strains were all Gram-positive spore-formers and were designated as CS201, CS202 and CS203, respectively. Other strains, including two *Lactobacillus* species, did not show any amylolytic activity. The amylolytic isolates were subsequently identified on the basis of their biochemical and cultural properties.

CS201 had wide, spreading, dull-surfaced colonies. The cells were short rods occurring in chains and with oval and central spores. The sporangium was not swollen. It was motile, oxidase-positive, catalase-positive, facultatively anaerobic, produced acid and gas from glucose, mannose and galactose, grew at pH 5.7 but not in 7% NaCl, was urease-negative and showed no growth at 60°C. The organism was identified as *Bacillus cereus*.

CS202 had cream, small, flat and round colonies. The cells were short motile rods occurring in chains. The spores were oval and central in non-swollen sporangia. The organism was oxidase-positive, catalase-positive, Voges Proskaeur reaction-positive, strictly aerobic, attacked glucose oxidatively with acid and gas production, showed no growth at 60°C, but grew in 7% NaCl and at pH 5.7. The organism was identified as *B. subtilis*.

CS203 had white, lobate, large and flat colonies. The cells were short motile rods. The spores were cylindrical and central in non-swollen sporangia. It

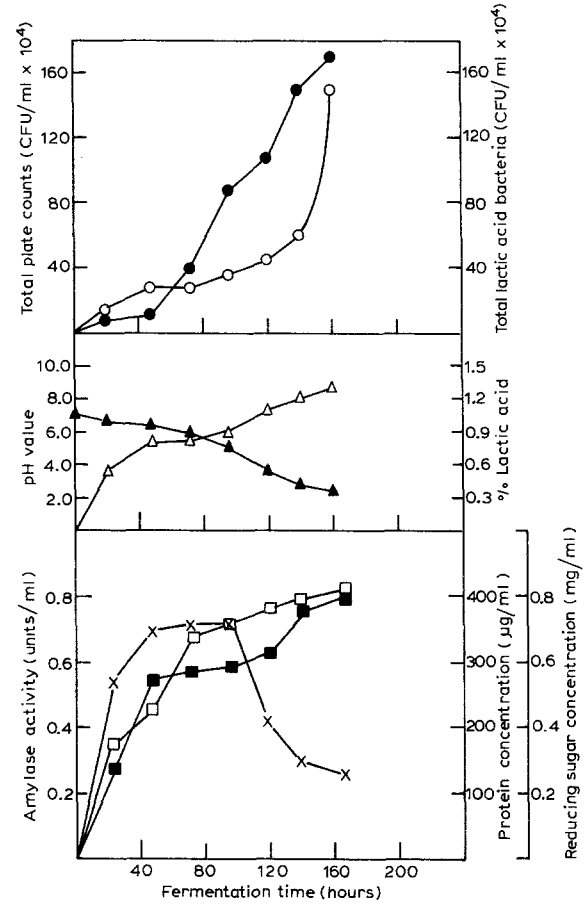

Fig. 1. Physico-chemical and microbiological changes occurring during cassava fermentation. ○, total plate counts; ●, total lactic acid bacterial counts; △, lactic acid concentration (%); ▲, pH value; X, reducing sugar concentration; □, amylase activity; ■, protein concentration. CFU = colony-forming units.

was catalase-positive, oxidase-positive, facultatively anaerobic, produced acid and gas from glucose, mannitol, arabinose and xylose, grew at pH 5.7 and in 70% NaCl but showed no growth at 60°C and was urease and nitratase-positive. The organism was identified as *B. licheniformis*.

Bacterial growth and amylase production

The production of extracellular amylase increased exponentially with growth in the three bacterial strains under standardized physical conditions until the stationary phase of growth was reached. *B. subtilis* grew on starch with a mean gen-

eration time of 2.74 h, whilst *B. licheniformis* and *B. cereus* grew with mean generation times of 2.54 and 1.08 h, respectively. Maximum growth was attained by the organisms after 24 h of incubation, with amylase production showing a little increase at this stage. However, *B. subtilis* produced relatively higher levels of amylase when compared to the other two species. The end-products of amylolysis in the three isolates were maltose and glucose, and their enzymes were probably α-amylases.

Effects of pH and temperature on enzyme activity

The three strains showed similar responses with respect to pH. The partially purified enzyme preparations of *B. subtilis* and *B. licheniformis* had their pH optima at 7.0 and 5.5, respectively, whilst *B. cereus* had its optimum pH at 7.5. With respect to temperature, *B. subtilis, B. licheniformis* and *B. cereus* had their optimum temperatures for amylase activity at 30°C, 37°C and 80°C respectively. The enzymes of *B. subtilis* and *B. licheniformis* were, however, still thermostable up to 75°C, after which their activities fell.

Effect of carbon sources on enzyme activity

The effect of carbohydrate substrates on amylase activity is shown in Table 1. There was no significant difference in amylase activities induced by either glucose or maltose and all the polysaccharides used.

DISCUSSION

The present investigation has indicated the involvement of amylase-producing bacteria in cassava fermentation. The bacterial isolates were predominantly *Bacillus* species and were different from those already reported by Collard and Levi [4] and Okafor [10]. The origin of these organisms might be cassava itself, or they may have been introduced during the steeping process.

The decrease in pH during cassava fermentation could be attributed to the steady increase in lactic acid concentration in the steeping liquor. The reducing sugar concentration, however, increased gradually during the first 72 h of fermentation, probably resulting from the action of amylolytic enzymes which hydrolysed the starch into reducing sugars. The decrease in the reducing sugar concentrations after 72 h could have resulted from its subsequent conversion into lactic acid by the *Lactobacillus* species that were present in the steeping liquor, since the reduction in reducing sugar levels coincided with a sharp rise in lactic acid concentration (Fig. 1).

The amylase produced by the *Bacillus* species isolated in this study were relatively less thermostable than those of other similar bacterial species reported in the literature. The amylase from strains of *B. licheniformis* were virtually unaffected by heat treatment, and in most instances the activity in-

Table 1

Effect of growth substrates on amylase production in broth cultures of cassava-fermenting bacteria

Substrate	Amylase activity (μg glucose/ml)			Protein concentration (μg/ml)			Specific activities (μg glucose/μg protein)		
	CS201	CS202	CS203	CS201	CS202	CS203	CS201	CS202	CS203
Glucose	1040	1090	1070	150.0	139.0	97.0	6.93	7.84	11.03
Maltose	760	760	820	96.0	88.0	85.0	7.92	8.64	9.65
Starch	1250	1040	980	120.0	82.0	104.0	10.42	12.68	9.42
Dextrin	1200	1020	1090	165.0	90.0	72.0	7.27	11.33	15.14
Amylopectin	940	880	970	86.0	102.0	66.0	10.93	8.63	14.69

creased slightly [8]. The amylases from *B. subtilis* and *B. amyloliquefaciens* showed reduced activity after incubation at 85°C for 60 min. Stark and Tetrault [11] showed that enzymes elaborated at thermophilic temperatures were more heat-stable than those elaborated at mesophilic temperature. The study of Campbell [3] also showed that amylases produced at thermophilic and mesophilic temperatures by the same strain of a facultative thermophile possess differences in thermal stability. The low thermal stability of amylases used in this work might therefore be due to the fact that they were elaborated at a temperature of 30°C.

The period of cassava fermentation for food processing could possibly be reduced by the seeding of pure cultures of amylolytic bacteria into the steeping liquor from the outset. The amylolytic bacteria isolated in this work could also be exploited on a large scale as sources of amylase.

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