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Comparative Production of Alpha-amylase, Glucoamylase and Protein Enrichment of Raw and Cooked Cassava by *Rhizopus Strains* in Submerged and Solid State Fermentations

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Three strains of the two species of *Rhizopus* were evaluated for production of alpha-amylase, glucoamylase and protein enrichment of raw and cooked cassava in submerged and solid state fermentations. In all the cases, the protein enrichment and enzyme production were higher in solid state fermentation, as compared to submerged fermentation. Similarly, cooked cassava showed higher productivities in solid state fermentation, except for higher production of glucoamylase by *R. oryzae* 28627 in solid state fermentation involving the use of raw cassava. The glucoamylase production by *R. oryzae* 28627 was 32-fold higher on raw cassava medium in solid state fermentation, as compared to that in submerged fermentation. Protein enrichment and alpha-amylase production were higher in cooked cassava medium, irrespective of the fermentation types employed. Data revealed the potential of *R. delamer* 34612 and *R. oryzae* 28168 for protein enrichment and alpha-amylase production, respectively, in solid state fermentation of cooked cassava. *R. oryzae* 28627 proved best for glucoamylase production in solid state fermentation of raw cassava.

Keywords : Raw cassava, Cooked cassava, Protein enrichment, Alpha-amylase production, Glucoamylase production, *Rhizopus*, Submerged fermentation, Solid state fermentation.

The genus *Rhizopus* represents an industrially important group of fungi for production of enzymes, such as lipases and glucoamylases (Djien 1984), and improvements in the nutritive quality of fermented foods (Thambirajah 1989; Tuncel et al. 1990). The role of amylases from *Rhizopus* sp. was stated to be very important in the different manufacturing processes to produce the various alcoholic beverages (Indian jackfruit wine, *sake* or *kaffir* beer) used in the Eastern countries (Steinkraus 1983, 1987). *Rhizopus* species are also often present in the different starters used in food fermentations (known under a variety of names, such as *murcha* in North India and Nepal, *ragi* in Indonesia, *look pang* in Thailand, *budbod* in the Philippines, and *chiu-chiu* in Taiwan and China in the Asian countries (Hesseltine et al. 1985). Since several hundred years, especially in countries such as China, Japan, Indonesia and Malaysia, *Rhizopus* species have been used in the preparation of various fermented foods (Djien 1984; Sasson 1988) such as the Indonesian *tempeh* (made from soybeans (*kedele*), groundnuts (*ontjam*), coconuts (*bongkrek*), the Chinese and Japanese *shoyu* (the fermentation of steamed soybeans and roasted ground wheat grains), the Japanese *miso* (whole fermented soybeans) and the Chinese *sufu*(a cheese-like product).

Fujio et al (1984) have reported several strains of *Rhizopus* sp. and *Rhizopus javanicus* IFO for their ability to produce ethanol directly from raw cassava starch. Ethanol (12.1g) was obtained after 12 days fermentation in flask at 35°C, when raw cassava was used at 40 g/l level. Subsequently, ethanol production between 13 and 14% after 4 days fermentation, with a yield of 72.3-83.5% (based on starch present initially in the medium), was achieved with the use of *Rhizopus koji* with raw cassava starch in a gas circulation type fermenter (Fujio et al. 1984). Many reports are available in literature on the capability of *Rhizopus* to hydrolyze starch from raw cassava. Nevertheless, the information on the efficiency of the different fermentation types and the strains used for such a bioconversion for metabolic production are scarce. The present investigation was, therefore, undertaken with a view to compare the efficiency of cassava fermentation by *Rhizopus* strains for enzyme production and protein enrichment by solid state and submerged fermentation systems.

Microorganisms : Three *Rhizopus* strains were studied. *Rhizopus oryzae* MUCL 28168 and MUCL 28627 were kindly supplied by Professor G.L. Hennebert, Mycology Collection, Catholic University of Leuven, Leuven, Belgium. *Rhizopus delemar* ATCC 34612 was obtained from the American Type

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Culture Collection, Rockville, Maryland, U.S.A. All the strains were maintained by periodic sub-culturing on potato-dextrose-agar (PDA) medium (Difco Laboratories, Detroit, Michigan, USA).

Sporangiospore production : Sporangiospores were produced on PDA medium in petri dishes after 8 days culture at 28°C. These were collected with a platinum spatula and transferred into tubes containing 20 ml of sterile physiological saline solution containing 0.01% Tween 80 and glass balls for vortexing so as to release maximum sporangiosphores and distribute these uniformly.

Preparation of raw cassava : Raw cassava was cultivated and harvested in Congo. Raw cassava was cut into small pieces (2-10 mm) and dried to 10% moisture content in forced air circulation drier at 50°C. The dried cassava pieces were ground and sieved to collect the fraction, containing granules of 0.8 to 2.0 mm size for use in fermentation.

Nutrient solution preparation: One hundred g raw cassava powder was mixed with a solution containing 4.75 g KH_2PO_4, 9.30g $(NH_4)_2SO_4$, 2.3 g urea, 2 x 10^9 spores and 96 ml water. The pH of the solution was adjusted to 5.0 with 5 N ammonia solution.

Preparation of cooked cassava : Dried raw cassava pieces were moistened to 40% level with the nutrient solution (as described above, but without spores) and heated to 120° C in an autoclave for 20 min. After cooling to 30°C, it was remoistened to 50% level with the nutrient solution, containing 2 x 10^9 sporangiospores of the culture.

Solid state fermentation : Solid state fermentation was carried out in a fermenter with an opening at the top for gas exchanges (Soccol 1992). Perforated polypropylene containers (diam 15.5 cm, depth 3 cm) containing 20 g fermenting cassava were placed on a perforated porcelain support. The culture system was placed in a temperature-controlled incubator for 48 h at 35°C (Fig. 1).

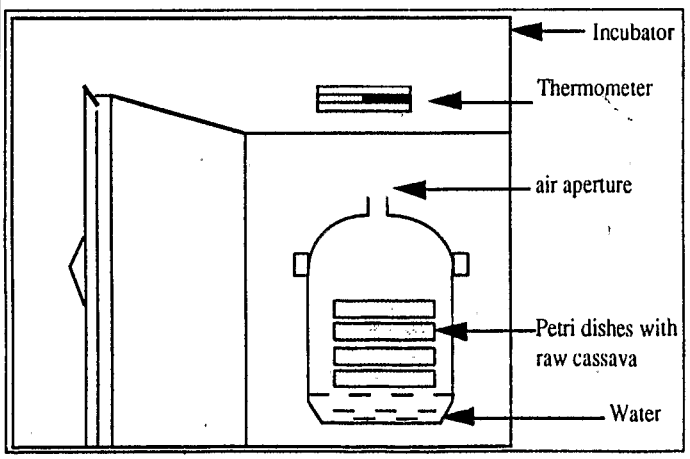

Fig 1. Incubation of *Rhizopus* strains in a moist atmosphere

Submerged fermentation : Dry cassava powder (granule size not exceeding 0.5 mm) was sterilized by a dry process for 24 h at 110°C, and 30 g of it was homogeneously dispersed in 1 l sterile salt solution and the pH was adjusted to 5.5 with 1 N NaOH. The composition of the sterile salt solution used was (g/l) : $(NH_4)_2SO_4$, 2.93; KH_2PO_4, 1.50; and urea, 0.72. Under aseptic conditions, the mixture was distributed in 50 ml quantity into 250 capacity Erlenmeyer flasks. The medium was inoculated with 2 x 10^7 spores /g dry cassava. When cooked cassava was used, the flasks were first autoclaved for 20 min at 120°C and cooled at 30°C before inoculation with the same amount of spores. The inoculated flasks were then placed on rotary incubator (model G-25 Shaker Incubator, new-Brunswick Scientific Co. Inc., Edison, U.S.A.) for 48 h at 35°C, the rate of rotation being 120 rpm.

Analytical methods : Fermenting medium (5 g or 5 ml in case of solid state or submerged fermentation) were removed and added to 50 ml distilled water. The mixture was blended in an Ultra-Turrax homogenizer (Janke and Kunkel GmbH, staufen i. Br., FRG) for 3 min at 20,400 rpm. It was immediately centrifuged under cold condition for 10 min at 6,000 rpm (6,120 **G**). Measurement of alpha-amylase activity was carried out in test tubes by adding 0.2 ml of supernatant to 1.6 ml of 1% soluble starch solution (pH 6.5) in a buffered phosphate solution. Incubation was performed at 40°C for 30 min and the reaction was stopped by addition of 0.2 ml NaOH (1N). The measurement of alpha-amylase activity was done by estimating residual starch content with iodine. One alpha-amylase unit is defined as the amount of enzyme that hydrolyzes 10 mg of starch in 30 min under the conditions used (Oteng-Gyang, 1979). For glucoamylase estimation, 0.2 ml of supernatant was added to 1.6 ml of 1% soluble cassava starch solution (pH 5.0) prepared in a buffered phosphate solution. Incubation was performed at 50°C for 60 min and the reaction was stopped by adding 0.2 ml NaOH (1N). Glucoamylase activity was determined by measuring reducing sugars released using the method of Miller (1959). One glucoamylase unit is defined as the amount of enzyme which releases

1 µmol glucose equivalent per min under the assay conditions. Proteins were determined using Lowry's method (Lowry et al. 1951). Samples were diluted 500 times with distilled water and homogenized using an Ultra-Turrax blender (at 20,000 rpm for 3 min) and a Potter homogenizer (Bioblock, Illkirch, France). Bovine serum albumin was used as standard. The values reported in all the cases are the average of a series of three fermentations.

Alpha-amylase production : Data indicated higher production of alpha-amylase in solid state fermentations by *Rhizopus* strains in case of both raw and cooked cassava, except for slightly higher enzyme production in submerged fermentation by *R. oryzae* 28168 in raw cassava medium (Table 1). *R. oryzae* 28627 produced highest enzyme in raw cassava medium, while *R. oryzae* 28168 produced highest enzyme in cooked cassava medium in solid state fermentation. Alpha-amylase production in cooked cassava medium was 2 to 4-folds higher by all the cultures, irrespective of the type of fermentation. Among all the cultures evaluated, *R. oryzae* 28168 appears to be the best for alpha-amylase production by solid state fermentation of cooked cassava (Table 1). Besides, *R. oryzae* alpha-amylase was produced more, when the decreasing level of substrate, such as starch, cellulose, pepper extract and glucose was used (Ekundaya et al. 1991).

Glucoamylase production : The data showed similar trends in case of this enzyme production by these two fermentation types. Glucoamylase production was 6-15 times higher in raw and cooked cassava medium under solid state fermentation, as compared to submerged fermentation (Table 1). However, raw cassava proved better than cooked cassava for producing higher amyloglucosidase titres, irrespective of the fermentation type. *R. oryzae* 28627 produced highest enzyme titres in solid state fermentation of raw cassava.

Proportion of alpha-amylase and glucoamylase titres : It is interesting to compare the proportion of these two enzymes produced by three different strains of *Rhizopus* as reported by Fujio et al (1984). In their studies, the titres of alpha-amylase and glucoamylase after 8 days of fermentation were reported to be 40.5-64.4 and 777.3-3113.5 U/g dry matter, respectively. The proportions of these enzymes observed in the present studies are entirely opposite, as all the three strains produced higher proportion of alpha-amylase than glucoamylase in case of submerged and solid state fermentation of raw and cooked cassava, except for higher production of gluco-amylase by *R. oryzae* 28168 and 28627 in solid state fermentation of raw cassava. The data indicate that the proportion of these two enzymes is governed by the strain used, nature of the substrate and fermentation type.

Protein enrichment : The data revealed that the protein enrichment of cassava was only 3.9-4.6% in submerged fermentation of raw cassava (Table 1), which improved to 9.3-10.8 % (slightly more than two-folds), when cooked cassava was used in place of raw cassava in submerged fermentation. In contrast, the protein enrichment was 10.5-11.0 %, even when raw cassava was used in solid state fermentation (Table 1). The data thus indicate the potential of solid state fermentation technique to give slightly higher protein enrichment, even with the use of raw cassava, as compared to submerged fermentation of cooked cassava. The economic gains with the use of solid state fermentation are thus apparent due to the elimination of cost-intensive cooking of cassava, which otherwise is essential with the use of submerged fermentation. The substitution of raw cassava by cooked cassava in solid state fermentation leads to further increase in the level of protein enrichment, but only marginally (Table 1), the range being 12.3 to 14.1 % and the increase amounting to 11.82 to 31.43%.

TABLE 1. COMPARATIVE PRODUCTION OF AMYLOLYTIC ENZYMES AND PROTEIN ENRICHMENT BY *RHIZOPUS* IN SUBMERGED AND SOLID STATE FERMENTATIONS OF RAW AND COOKED CASSAVA

Product	Substrate	Fermentation type	Final culture		
			Rhizopus oryzae 28168	*Rhizopus oryzae* 28627	*Rhizopus delemar* 34612
Alpha-amylase	Raw Cassava	SmF	42.2	76.0	40.4
		SSF	39.3	98.0	55.0
	Cooked Cassava	SmF	157.2	145.4	168.5
		SSF	178.4	167.0	170.0
Gluco-amylase	Raw Cassava	SmF	9.6	7.8	7.3
		SSF	55.3	108.0	70.0
	Cooked Cassava	SmF	3.1	3.3	5.7
		SSF	46.2	37.0	47.0
Protein	Raw Cassava	SmF	3.9	4.0	4.6
		SSF	11.0	10.5	11.0
	Cooked Cassava	SmF	10.0	9.6	9.3
		SSF	12.3	13.8	14.1

SmF : submerged fermentation,
SSF : solid state fermentation

The titres of the enzymes (the units) are per 1 g or 1 ml of fermenting medium in case of solid state and submerged fermentations, respectively. The protein production reported is g/100 g or ml of the medium.

Among all the fermentation studies, the highest protein enrichment (14.10%) was shown by *R. delemar* 34612 in solid state fermentation of cooked cassava. The protein enrichment in case of solid state fermentation of raw cassava was in the range of 10.53-10.96% for all the three culture studies.

It is interesting to note that the trends of results observed in the present studies are similar to those reported for *Aspergillus niger* (Alazard and Raimbault 1981). The results indicate the significant importance of the mode of cultivation in the fungal biosynthesis of some very important industrial enzymes. In addition, these indicate the potential of *Rhizopus* in protein enrichment of cassava by solid state fermentation of raw or cooked cassava. The same rationale is true for the production of different metabolites by *Rhizopus* in the same fermentation type. The most important aspect of the results described in this paper relates to the direct use of raw cassava, thereby eliminating the cooking step, which otherwise is essential for gelatinization and liquefaction of starch (about 80% of the dry matter) present in cassava. By depending on this approach, all the problems related to thermic treatment of cassava are solved and the cost is also reduced, in addition to saving of much needed energy.

The trends of the results indicate the need for further work on the feasibility of utilizing raw cassava as an efficient substrate for production of amylolytic enzymes or protein enrichment by *Rhizopus*. Raw cassava could prove an inexpensive source of starch and constitute a strategic biological matter for production of commercially valuable microbial metabolites.

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