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ORIGINAL PAPER

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Breeding and growth of *Rhizopus* in raw cassava by solid state fermentation

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Abstract Nineteen *Rhizopus* strains were selected and tested for their growth capacity on raw cassava starch and their ability to produce amylase when grown on solid-state fermentations. Only three strains grew significantly on this natural substrate. Glucoamylase production was higher on raw cassava than on cooked cassava. After 48 h of fermentation, the protein content of cassava was increased from 1.75% to 11.3%. The by-products of fermentation were fumaric acid, lactic acid and ethanol.

Introduction

Rhizopus spp. are microscopic filamentous fungi of the order Mucorales. They belong to the Phycomycetes (primitive fungi), subclass Zygomycetes (Onions et al. 1981; Fassatiova 1986; Samson and van Reenen-Hoekstra 1988). Several *Rhizopus* species are of considerable interest for the food industry. They have been used in solid-state fermentation for several centuries, especially in Asia (China, Korea, Japan, Indonesia, Malaysia, Singapore, Java, etc.) for preparing many fermented foodstuffs (Hesseltine 1965; Raimbault 1981). *Rhizopus* not only enhances the digestibility and protein content of foodstuffs (Soccol et al. 1992; Soccol 1992; Cook 1982; Beuchat 1987), but also prevents the formation of toxic substances such as aflatoxin B_1 (Ko 1988). *Rhizopus* spp. can produce numerous anti-carcinogenic substances (Zhu et al. 1989), synthesize an antibiotic that is extremely active against a large number of Gram-positive bacteria (Wang et al. 1969) and detoxify cassava cyanogenic glycosides (linamarin) (Padmaja and Balagopal 1985).

Cassava (*Manihot esculenta* Grantz) is the staple food over 500 million people in Latin America, Asia and Africa (Cook 1982). However, the protein and vitamin contents are low. A diet of cassava alone causes chronic deficiency-related diseases in the long term. A method for increasing significantly the growth of certain *Rhizopus* strains on raw cassava in solid-state fermentation is described here. It leads to a set of simple applications for appreciable improvement of quality of cassava meal (Soccol et al. 1992; Soccol 1992).

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Materials and methods

Micro-organisms

Nineteen *Rhizopus* strains were studied (Table 1). Some strains were obtained from various international collections [American Type Culture Collection (ATCC), Rockville, Md., USA] and others were kindly provided by Dr. C. W. Hesseltine and Dr. Kurtzman [Northern Regional Research Laboratory (NRRL), U.S. Department of Agriculture, Peoria, Ill., USA] and by Prof. G. L. Hennebert [Mycology Collection (MUCL), Catholic University of Leuven, Leuven, Belgium]. The origins of the strains are reported in Table 1.

Sporangiospore production

Sporangiospores of the different strains tested were produced on potato dextrose agar (PDA) medium in petri dishes after culture for

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8 days at 28°C. Spores were collected from the petri dishes using a platinum rod and suspended in tubes each containing 20 ml of sterile physiological saline solution with 0.01% Tween 80 and glass balls. Extraction was enhanced by agitation.

Origin of the plant material

Cassava roots (*M. esculenta* var. Ngansa) were harvested in the Brazzaville area 15 months after planting. After washing, peeled roots were cut into small pieces and dried. The dried material was preserved under sterile conditions in a plastic bag. All the experiments were carried out on this material in our laboratory.

Preparation of raw cassava

Just before using, the dried raw material was ground. After sieving, the 0.8–2.0 mm fraction was used for fermentation. At this stage, it had a 10% moisture content and contained 82% starch on a dry weight (DW) basis. A saline solution containing 4.75 g KH_2PO_4, 9.30 g $(NH_4)_2SO_4$, 2.3 g urea and 96 ml water (adjusted to pH 5.0 with a solution of 5 M ammonia) was mixed with 100 g raw cassava meal. The mixture was inoculated with 2×10^9 spores. At this stage of the preparation, the initial pH was reported as 5.8.

Preparation of cooked cassava

Raw cassava was moistened to 40% using the saline solution described above. It was then heated to 120°C in an autoclave for 20 min, cooled to 30°C and remoistened to 50% with the same saline solution and then inoculated.

Method of cultivation

Cultures were carried out in a glass incubator with an opening at the top for gas exchange. Perforated polypropylene containers (15.5 cm in diameter, 3 cm in depth) containing 20 g cassava were placed on a perforated porcelain support. The incubator was placed in a thermostatically controlled oven for 48 h (Fig. 1).

Treatment and analysis of the samples

Dry weight

At the end of the fermentation, each container was drained off in a 500-ml beaker. The fermented meal was carefully homogenized with a spatula. The resulting mixture (M_1) was weighed in an aluminium dish (Fig. 2). A sample weighing about 5 g was dried in an oven for 24 h at 105°C and then cooled in drier containing silica gel for calculation of the DW.

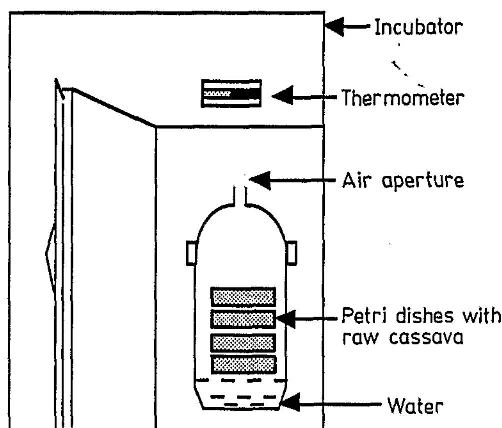


Fig. 1. Incubation of *Rhizopus* strains in a moist atmosphere

pH

The pH was measured in a mixture (M_2) obtained by homogenisation of a 5-g sample diluted in 50 ml distilled water using an Ultra-Turrax at 20,400 rpm for 3 min.

Enzymatic activity

Measurement of α-amylase activity was performed in test tubes by adding 0.2 ml supernatant to 1.6 ml cassava starch solution prepared at 10 g/l and adjusted to pH 6.5 using a buffered phosphate solution (1/15 M KH_2PO_4, 6.389 g/l; $Na_2HPO_4 \cdot 2H_2O$, 3.5195 g/l). Incubation was performed at 40°C for a maximum of 30 min. The reaction was stopped to measure α-amylase activity. One α-amylase unit (u) is defined as the amount of enzyme hydrolysing 10 mg starch in 30 min under the conditions described by Oteng-Gyang (1979).

For glucoamylase activity, 0.2 ml supernatant S_1 was added to 1.6 ml soluble cassava starch solution at 10 g/l buffered to pH 5.0 using a phosphate solution (1/15 M KH_2PO_4, 9.0 g/l; $Na_2HPO_4 \cdot 2H_2O$, 0.095 g/l). Incubation was performed at 50°C for a maximum of 60 min. The reaction was stopped by adding 0.2 ml of 1 M NaOH. Glucoamylase activity was determined by measuring release of reducing sugars using the method of Miller (1959). Sugars are expressed as glucose equivalents. One glucoamylase unit is defined as the amount of enzyme that releases 1 μmol glucose/min (Alazard and Raimbault 1981).

Proteins

Proteins were determined using the method of Lowry et al. (1951) on fraction M_3.

Sugars

Total sugars were determined after acid hydrolysis by addition of a solution of 3 M HCl for 30 min at boiling point. Hydrolysed reducing sugars were measured using the method of Miller (1959).

Metabolites

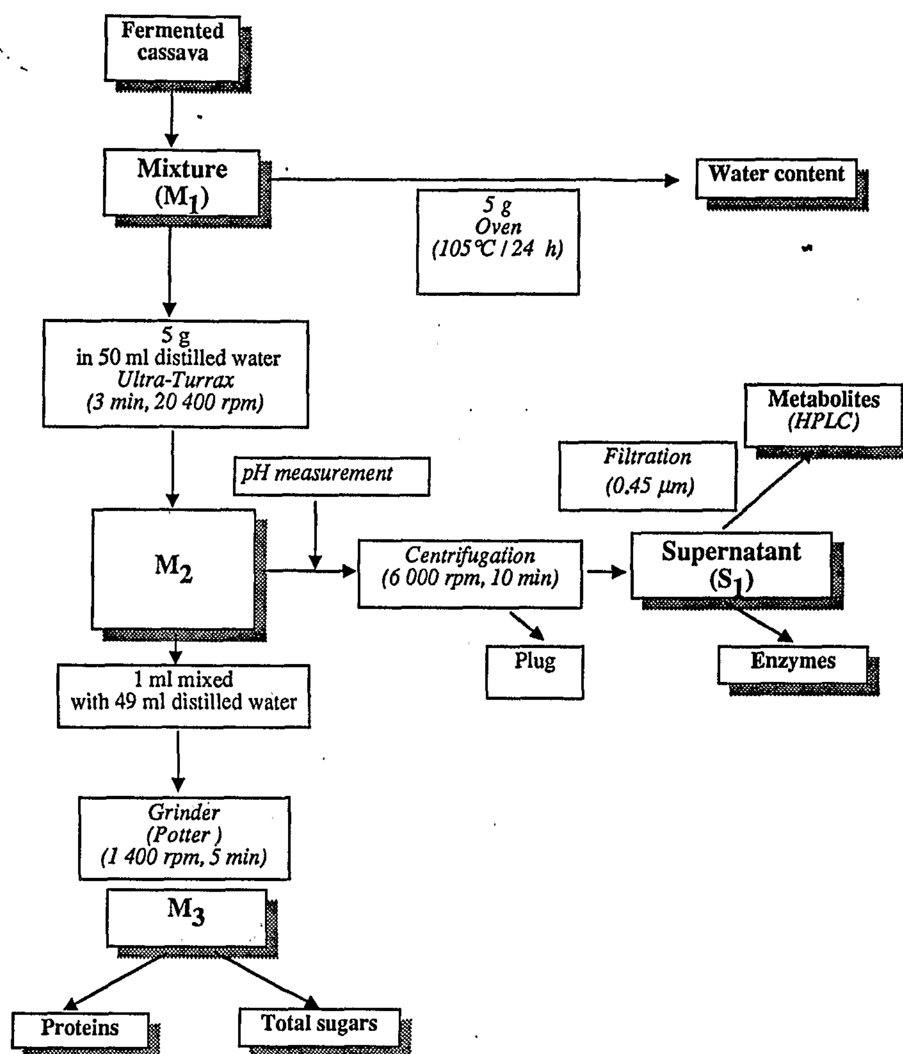
The metabolites analysed were separated and determined by HPLC on an Aminex HPX 87H column (Bio-Rad Laboratories, Paris, France) according to Giraud et al. (1991). The results are the average of a series of three fermentations. Each analysis was performed in triplicate.

Results

Screening of strains

Growth data for the various *Rhizopus* strains studied are reported in Table 1. Thus, only three of the 19 strains selected grew significantly on raw cassava (*R. oryzae* MUCL 28168, *R. delemar* ATCC 34612 and *R. oryzae* MUCL 28627). In contrast, strains

Fig. 2. Preparation of the samples for analysis



R. oligosporus NRRL 2710, *R. microsporus* MUCL 9667, *R. delemar* NRRL 1472 and *R. circicans* NRRL 1475 were unable to use raw cassava starch as the sole carbon source.

Fumaric acid, lactic acid and ethanol were the main metabolites produced during growth. The organic acids produced during growth were responsible for the decrease in pH of the mixture, especially in the case of the three strains able to produce these metabolites.

R. delemar ATCC 34612 gave the best enrichment in proteins of cassava meal. The best result was 10.96 g protein/100 g DW cassava meal. In parallel, the sugar consumption was 28.75%. The ratio of protein formed to sugar uptake was 39.59%.

Effects of cooking

The best growth on raw cassava was obtained with *R. oryzae* MUCL 28168, *R. delemar* ATCC 34612 and *R. oryzae* MUCL 28627, which were therefore chosen for more detailed studies of the ability to break down cassava starch. Experiments were carried out to compare growth and enzyme production on raw and cooked cassava. Growth was followed by protein enrichment, which reached 11–14% (Table 2). It was 10–11% in raw cassava and reached 12–14% in cooked cassava. Nevertheless, the results for all the strains tested and activities monitored were different according to the substrate used. Thus, α-amylase activity was higher in cooked cassava. The maximum α-amylase activity of *R. oryzae* MUCL 28168 was 178.40 U/g DW cassava. In contrast, glucoamylase activity was slightly greater for all the strains cultured on raw cassava. The highest glucoamylase activity was 108 U/g DW with *R. oryzae* MUCL 28627 on raw cassava.

Effect of different culture conditions

Figure 3A shows the effect of temperature on protein synthesis, α-amylase and glucoamylase activities during

Table 1. *Rhizopus* strains able to breakdown raw cassava in a solid medium

Strain	Raw cassava breakdown	Protein (g/100 g DW)	Total sugars (g/100 g DW)	pH	Metabolites (g/100 g DW)
R. stolonifer MUCL 28169	++	6.70	68.10	5.68	Lactic acid (1.08)
R. oryzae NRRL 695	+	3.41	75.18	6.43	Lactic acid (1.55)
R. oligosporus NRRL 2710	−	2.61	76.78	6.23	—
R. microsporus ATCC 46436	++	6.45	69.15	5.65	Fumaric acid (2.84)
R. arrhizus MUCL 28425	+	2.98	75.54	6.39	Fumaric acid (1.23) Lactic acid (0.89)
R. oryzae NRRL 25976	+	3.38	75.52	6.42	Fumaric acid (0.35) Lactic acid (0.32)
R. microsporus MUCL 9667	−	2.51	76.98	6.17	—
R. arrhizus MUCL 16179	+	3.18	75.16	6.85	Lactic acid (1.84)
R. arrhizus NRRL 1526	+	3.62	74.15	7.46	Lactic acid (1.42)
R. oryzae MUCL MUCL 28168	+++	10.93	60.14	4.97	Fumaric acid (3.56)
R. formosa MUCL 28422	++	6.40	69.20	6.36	Lactic acid (3.21) Ethanol (1.25)
R. delemar ATCC 34612	+++	10.96	58.08	4.16	Lactic acid (3.68) Fumaric acid (7.13) Ethanol (2.95)
R. delemar NRRL 1472	−	1.75	79.77	7.10	—
R. sp. NRRL 25975	++	5.79	70.42	5.18	Fumaric acid (4.16) Ethanol (0.66)
R. oryzae MUCL 28627	+++	10.53	58.15	5.08	Fumaric acid (4.90) Ethanol (1.95)
R. oryzae ATCC 22580	++	5.27	73.46	6.12	Fumaric acid (1.25) Lactic acid (0.39)
R. solonifer MUCL 28181	+	3.47	74.58	6.66	Lactic acid (0.33)
R. circicans NRRL 1475	−	1.87	80.31	7.10	—
R. oligosporus ATCC 6203	++	5.41	70.70	4.93	Fumaric acid (2.55) Lactic acid (1.89)
Raw cassava	−	1.75	81.52	5.60	—

ATCC, American Type Culture Collection (Rockville, Md., USA); MUCL, Mycology Collection, (Catholic University of Leuven, Leuven, Belgium); NRRL, Northern Regional Research Laboratory (U.S. Department of Agriculture, Peoria, Ill., USA): −, no growth; +, growth; ++, good growth; +++, excellent growth; DW, dry weight

Table 2. Effect of cooking on protein synthesis and production of α-amylase and glucoamylase by different *Rhizopus* strains cultured in solid medium

	Raw cassava			Cooked cassava		
Strain	α-Amylase (Ug/DW)	Glucoamylase (Ug/DW)	Protein (g/100 g DW)	α-Amylase (U/g DW)	Glucoamylase (U/g DW)	Protein (g/100 g DW)
R. oryzae MUCL 28168	39.30	55.30	10.96	178.40	46.22	12.30
R. delemar ATCC 34612	55.00	70.00	10.93	170.00	47.00	14.10
R. oryzae MUCL 28627	98.00	108.00	10.53	167.00	37.00	13.80

U, Units

the growth of *R. delemar* ATCC 34612 on raw cassava in solid medium. The decrease in α-amylase activity observed was not significantly affected by temperature and remained fairly constant throughout incubation. In contrast, a temperature of 30 or 35°C was more favourable for glucoamylase synthesis. Temperature should therefore be taken into account in this type of fermentation. Under the best conditions at 35°C, growth was optimal with a high protein content (11.2%) and considerable α-amylase and glucoamylase activities. Decrease in growth and in enzyme production was observed at 40°C.

The moisture content affected growth of *R. arrhizus* ATCC 34612 cultured on raw cassava pellets. Figure 3B shows that an initial water content of 50–52% gave the highest α-amylase and glucoamylase activities and the highest protein contents. At less than 45% and above 55% moisture content, the growth, protein content and α-amylase and glucoamylase activities were decreased.

Spore inoculation must be sufficient to ensure homogeneous seeding of substrate and rapid starting of growth to prevent possible competition with contaminants, particularly in the case of raw cassava that has not been subjected to heat treatment. The appropriate

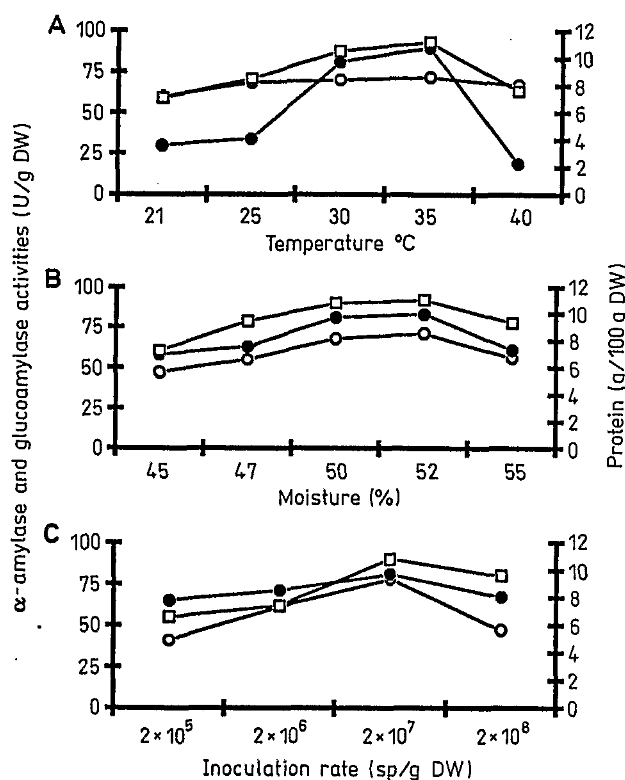


Fig. 3A–C. Effect of different culture conditions on protein synthesis (□) and production of α-amylase (○) and glucoamylase (●) by *Rhizopus delemar* ATCC 34612 on raw cassava in solid medium. **A** Temperature, **B** Moisture, **C** Inoculation rate; sp. spores; DW, dry weight

quantity of spores to be added per gram DW raw cassava was therefore calculated. The results in Fig. 3C show that growth and production of α-amylase and glucoamylase activities were higher with inoculation of approximately 2×10^7 spores/g DW. In contrast, a higher or lower level caused a decrease in fungus growth and enzyme synthesis.

Growth kinetics on raw cassava

Figure 4 shows the growth kinetics of *R. delemar* ATCC 34612 cultivated on raw cassava inoculated with 2×10^7 spores/g DW moistened to 50% and incubated at 35°C for 48 h. The results show that protein production and enzyme synthesis became significant after 16 h of fermentation (Fig. 4A and B). Synthesis of α-amylase and glucoamylase increased steadily until the end of fermentation (Fig. 4B). The quantity of protein synthesised during growth increased linearly between 16 and 40 h (Fig. 4A). The protein content of raw cassava meal reached 11.3% after 48 h of fermentation with a 41.52% yield based on sugar uptake.

Glucose accumulation was observed during fermentation. It reached 18 g glucose/100 g dry raw cassava (Fig. 4C). Fumaric acid and ethanol were produced during growth, reaching 4.2 g/100 g DW and, 1.8 g/100 g DW, respectively (Fig. 4C). A slight increase in moisture content from 50 to 55% and a decrease in pH from 5.8 to 3.8 was recorded, probably due to the production and the accumulation of fumaric acid (Fig. 4D).

Discussion

It was shown during this work on the choice of *Rhizopus* strains able to grow on raw cassava starch that this substrate was not necessarily used by all the tested strains. Of the 19 strains of various species, only three grew significantly on the substrate used as a solid medium (*R. oryzae* MUCL 28168, *R. delemar* ATCC 34612 and *R. oryzae* MUCL 28627).

This study on the ability of different strains of *Rhizopus* to grow and produce amylases under different culture conditions on solid medium, and especially on raw and cooked cassava, shows that α-amylase activity was higher on cooked than on raw cassava. However, glucoamylase activity was higher on raw cassava for all the strains tested. The results can be compared to those of Fujio et al. (1984) on *koji* using similar micro-organisms; the authors reported 40.5–64.4 U/g DW α-amylase activity and 777.3–3313.5 U/g DW glucoamylase activity after 1 week of culture. Our results are comparable for α-amylase activity but lower for glucoamylase activity. The higher glucoamylase figures reported by Fujio et al. (1984) result probably from the nature of the substrate and the duration of culture.

The protein content was higher for cooked cassava than for the raw preparation. However, considerable protein enrichment (10–11%) was obtained with raw cassava; this is not negligible. The figures are comparable to those of Daubresse et al. (1987) for strains of *R. oryzae* MUCL 28627 cultured on cassava steam-cooked on trays.

Study on the effects of different culture conditions on the growth and production of amylases by *R. delemar* ATCC 34612 enabled us to define optimal conditions for the growth of *Rhizopus* on raw cassava: temperature, 30–35°C; moisture content, 50–52%; inoculation level, 2×10^7 spores/g DW. These figures confirm the results for *Aspergillus niger* cultured on gelled cassava starch (Raimbault 1981, 1989; Oriol et al. 1988a).

The growth kinetics of *R. delemar* ATCC 34612 on raw cassava lead to a balanced product. Fermentation for 48 h is sufficient for obtaining meal with an excellent appearance and a protein content of about 11.3%. Glucose accumulation in relation to hydrolysis of raw cassava starch throughout growth of the fungus is a fundamental, innovative argument for improvement of the nutritional and industrial value of cassava meal. The slight increase in water content during growth after 48 h had previously been observed with *A. niger* cultured on

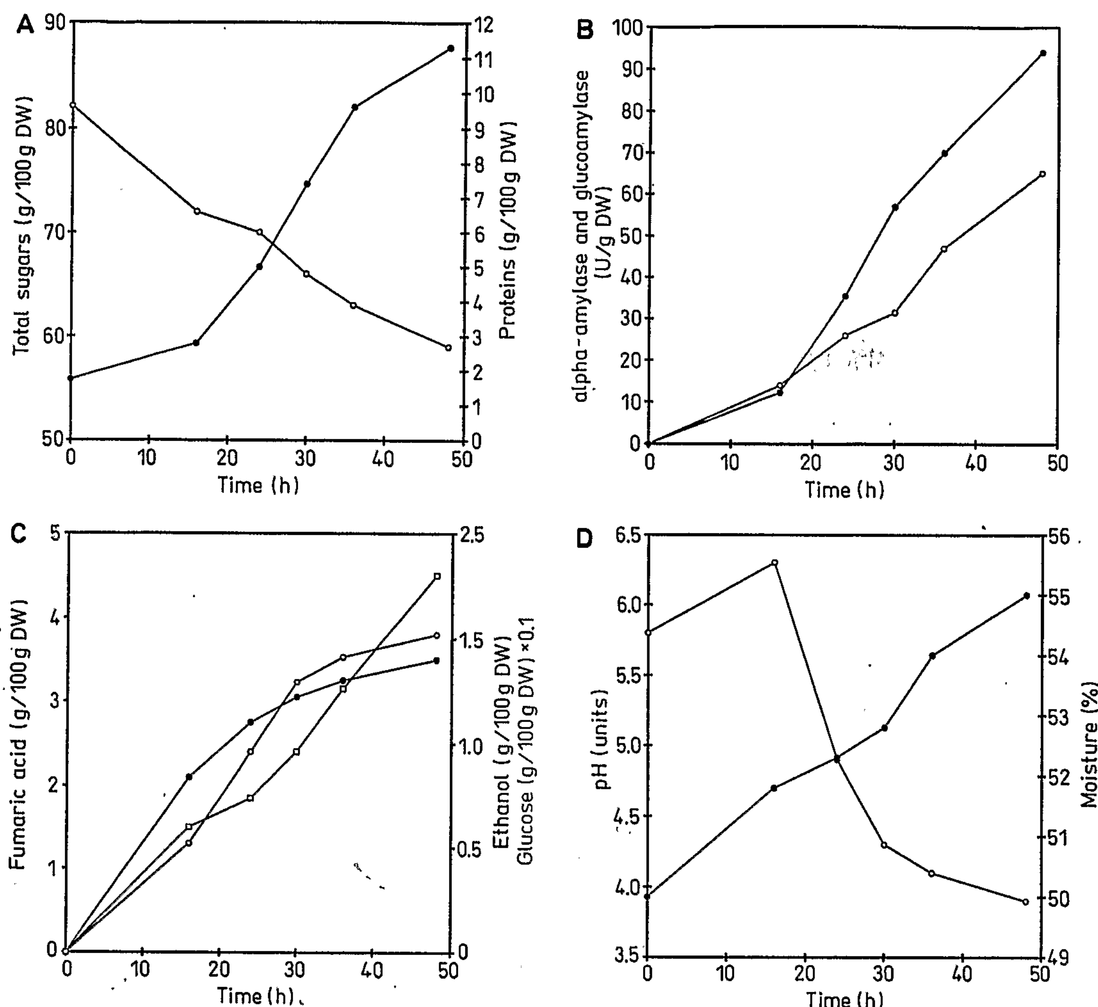


Fig. 4A–D. Growth kinetics of *R. delemar* ATCC 34612 on raw cassava in solid medium. **A** changes in total of sugar uptake (o) and protein production (o) with time. **B** changes in α-amylase (o) and glucoamylase (•) with time. **C** Glucose formation (□) and production of ethanol (•) and fumaric acid (o). **D** Changes in moisture context (•) and pH (o) with time

cooked cassava (Raimbault 1981, 1989; Oriol et al. 1988a, b).

The accumulation of fumaric acid observed after 16 h causes a decrease in pH; this is excellent for prevention of contamination by unwanted micro-organisms.

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