Growth of *Rhizopus* sp. on ungelatinized cassava flour in solid state fermentation for protein enrichment

M. RAIMBAULT AND C. RAMIREZ TORO

Laboratorio Bioconversìon, Dept. de procesos Quìmicos y Biològicos,
Fac. Ingenieria, Universidad del Valle, Apartado Aereo: 25 360 Cali, Colombia.

SUMMARY

In order to study the potential of bioconversion of crude cassava flour, without gelatiization, it was necessary to apply a preliminary treatment to eliminate the natural microflora (bacteria, yeast and fungal spores), without allowing starch gelatinization, which otherwise would occur in steam sterilization process. Studies on the effect of temperature and moisture content of flour on micro-organisms content and rate of starch gelatinization, allowed the optimization of a dry or semi-dry treatment of cassava flour to obtain a micro-organism-free ungelatinized crude flour. The pretreated flours, as such or after mixing (50/50) with crude soya flour, were inoculated with spores suspension of selected strains of *Rhizopus* sp. *(oryzae, arrhizus, oligosporus)*, and incubated in a column reactor, equipped for on line respirometric analysis in solid state fermentation. The efficiency of each strain, in terms of specific growth rate, protein enrichmen and sugars bioconversion, was determined. Crude cassava flour could be enriched with up to 14% of protein, in contrast to that of 20%, in case of 50/50 mixture of crude cassava and soya flours. The protein enrichment of cassava by *Rhizopus* sp. is of commercial importance due to GRAS clearance of this fungi.

Keywords: Solid state fermentation, *Rhizopus* spp., ungelatinized cassava flour, cassava+soya flours, column fermentors, on-line respirometry, bioconversion, protein enrichment.

RESUME

Culture de *Rhizopus* sp. sur de la farine de manioc non gélatinisée en fermentation en milieu solide pour l'enrihissement en protéines.

RAIMBAULT M. ET RAMIREZ TORO C.

Afin d'étudier les potentialités de bioconversion de farine de manioc non gélatinisée par *Rhizopus* sp., il a été nécessaire de mettre au point un traitement préalable permettant d'éliminer la microflore naturelle (bactéries, levures et spores de champignons) tout en évitant de réaliser une gélatinisation de l'amidon, comme c'est le cas pour le procédé de stérilisation à la vapeur. Après une étude sur l'effet de la température et de l'humidité de la farine sur le contenu en microrganismes, et sur le taux de gélatinisation, il a été possible d'optimiser un traitement de farine sèche ou semi-sèche, de façon à obtenir une farine crue propre, pour les études ultérieures avec *Rhizopus* sp. Les farines de manioc prétraitées ont été utilisées pures ou en mélange (50/50) avec de la farine crue de soja, puis inoculées avec des spores de souches sélectionnées de *Rhizopus* sp. (*R.oryzae, R.arrhizus, R.oligosporus*). Elles ont alors été incubées dans des réacteurs colonnes adaptées à la FMS et à la respirométrie en ligne. Les analyses biochimiques et chimiques initiales et finales ont été réalisées de façon à évaluer l'efficacité de chaque souche à transformer le manioc cru, en terme de croissance spécifique, d'enrichissement en protéines et de bioconversion des sucres. Les résultats indiquent qu'avec de la farine de manioc cru, il est possible d'atteindre 14% de protéines, alors qu'en mélange avec du soja (50/50) on peut obtenir jusqu'à 20% de protéines. Il est rappelé que *Rhizopus* est une moisissure bien connue pour la production d'aliment de bonne qualité pour l'alimentation humaine. De plus *Rhizopus* produit des métabolites d'intérêt alimentaire pour la conservation, la préservation et l'arôme des aliments.

Mots clés: Fermentation en milieu solide, *Rhizopus* sp., farine de manioc non gélatinisée, mélange de farines de soya et de manioc, fermenteurs colonne, respirométrie en ligne, bioconversion, enrichissement protéique.

INTRODUCTION

Studies on solid state fermentation (SSF) of fungi on cassava and amylaceous substrates have been carried out in ORSTOM for more than 15 years. Initially, the

protein enrichment of cassava and other tropical substrate, like cassava, potato and banana, was studied out, using fungi of the *Aspergillus* group (Raimbault, 1981). It was possible to obtain fermented cassava with 18-20% protein on a dry matter (DM) basis (Table 1). Applications were essentially oriented to animal feeding.

Table 1. Protein enrichment by *Aspergillus niger* after 30 h fermentation in solid state fermentation

SUBSTRATE	Initial composition		Final composition	
	Proteins	Sugar	Proteins	Sugar
Cassava	2.5	90	18	30
Banana	6.4	80	20	25
Banana refuse	6.5	72	17	33
Potato	5.1	90	20	35
Potato Waste	5.1	65	18	28

Recently, Soccol *et al* (1993) obtained good results with fungi of the *Rhizopus* group, of special interest in human traditional fermented foods. Work was carried out on the pretreatment, and the effect of cooking on starch availability, protein content and yield of the bioconversion of starch into protein (Table 2). Without any cooking, a selected strain of *Rhizopus oryzae* could transform cassava, containing only 1.68% protein, into fermented cassava, containing 10.89% protein. The results indicated possibility of protein enrichment of raw cassava meal, by using selected strains of *Rhizopus*, with ability to grow on crude ungelatinized starch (Table 2). The application of such fermented cassava was oriented to human consumption.

With respect to amylase biosynthesis, data showed that the amounts of $\alpha-$ amylase and glucoamylase were 10 to 15 times higher in the solid than in the liquid cultures. Moreover, these enzyme titres were higher in crude starch medium than in cooked cassava (Soccol *et al*, 1994b).

Table 2. Growth of *Rhizopus oryzae* in solid substrate fermentation on cassava granules after various treatment (Soccol *et al*, 1994b)

TREATMENT	TOTAL SUGAR		PROTEINS*		Y
	Initial	Final	Initial	Final	Pr/sugar
I	80,01	46,78	1,20	11,69	0,174
II	84,11	60,72	1,61	12,40	0,227
III	82,44	52,57	1,56	13,93	0,208
IV	82,49	56,62	1,47	11,89	0,215
V	82,04	56,62	1,68	10,89	0,190

I : Cassava, autoclaved 30 min, 120°C, freezed, dried, grounded

II : Cassava flour (40% water), autoclaved 30 min at 120°C

III: Cassava flour (30% water), autoclaved 30 min at 120°C

V : Crude cassava flour without treatment

* g / 100 g Dry Matter ; ** g / 100 g Total weight

The work is continued in an EEC program at the bioconversion laboratory of the Universidad del Valle, Cali, Colombia, with the particular view to enhance knowlege about specificity of strains of *Rhizopus*, which are able to degrade the crude granule of starch. This could simplify processing of cassava tuber, having an important ecological aspect, as the plant starch is present in ungelatinized crude form in the natural environment.

It was necessary to get purée flour of crude cassava. In fact, the commercial flour of cassava harbours a large number of natural contaminants to allow microbial studies with fungi, without conventional sterilization and the consequent gelatinization. Therefore, studies were conducted to obtain crude cassava meal, with low content of bacteria as well as fungi, and without significant gelatinization.

MATERIAL AND METHODS

A number of techniques allow to evaluate the gelatinization of starch: enzymatic sensibility to glucoamylase, enthalpy energy, viscoamylogram, crystallography and Lugol reaction. For routine purposes, it was necessary to develop a very simple and rapid method, in view of a large number of determinations. The method involved the use of glucoamylase to measure the efficiency of the hydrolysis of the starch, as an

index of starch gelatinization and proved to be time consuming and costly. Hence, the method of Wootton *et al* (1971) was preferred as it demostrated good correlation coefficient of the calibration curve in well standardized conditions (Fig. 1). This technique involved the measurement of the blue coloration developed by the Lugol reaction. Calibration of the intensity of the reaction, as a function of the extent of gelatinization of cassava starch, was reliable. Starch, with different extent of gelatinization, was obtained by mixing crude (0% gelatinization) cassava flour with well gelatinized (100%) cassava flour. The correlation (0.99) between the optical density at 600 nm and the degree of gelatinization indicated the validity of the technique.

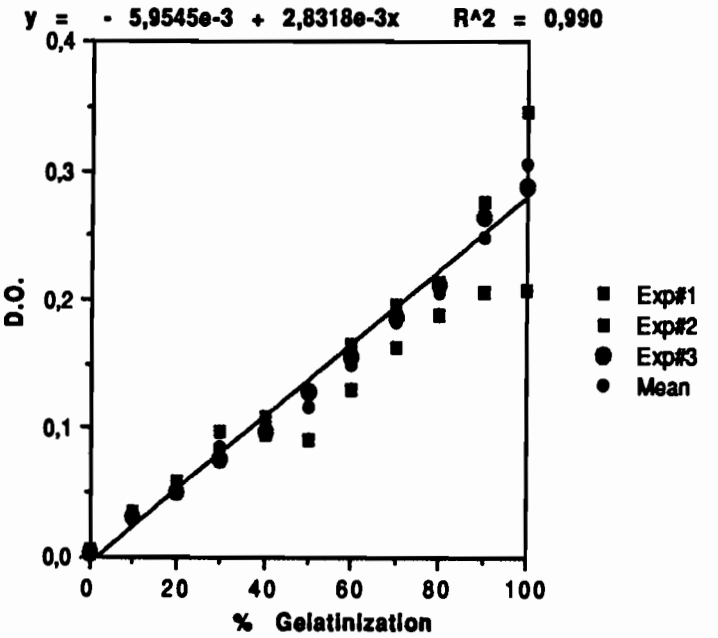

Fig. 1. Calibration curve for the determination of gelatinization ratio.

For the determination of the microflora, the UCP technique was employed using PCA medium for total microflora and PDA medium for spores and yeasts contents, as recomended by Mitchell *et al* (1988).

The technique for *Rhizopus* cultivation in solid state fermentation was described previously (Raimbault and Alazard, 1980). For the measurement of the respiratory metabolism, the automatic gas chromatographic column method was used, as described by Saucedo *et al* (1994).

Strains of *Rhizopus* were the same, as the ones used by Soccol *et al* (1994a): *R. oryzae* 28168; *R. oryzae* 28627; *R. arrhizus* 1526 and *R. oligosporus* 6203.

RESULTS AND DISCUSSION

EFFECT OF DIFFERENT TREATMENT OF DRY CASSAVA MEAL

Crude cassava meal contained about 108-109 bacteria/ g DM, which makes it difficult to develop studies for fermentation of ungelatinized starch in natural conditions. It is, therefore, necessary to reduce the level of bacterial and fungal population, in order to eliminate competition with the spore inoculum (107/ g). Fig. 2 indicates the effect of UV ray, temperature and microwave on the bacterial content of dry cassava meal of less than 10% moisture content.

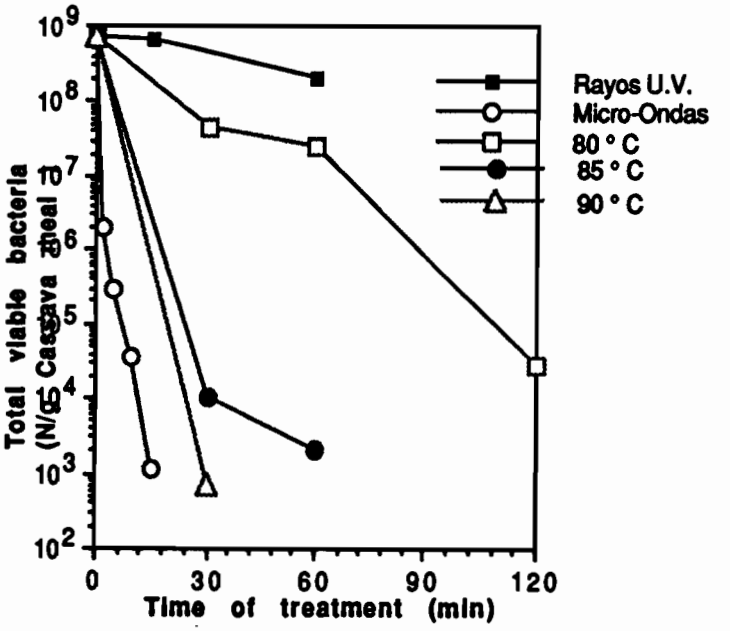

Fig. 2. Total bacterial microflora (PCA) in relation to the treatment and time.

The efffect of UV ray was poor and not sufficient enough to elimine the natural microflora, neither for bacteria, nor for yeast and fungi. On the contrary, microwave reduced fungal content from 108 to 103 / g after 2 min, and bacterial content from 8.108 to 103 bacteria / g after 2.5 min. Dry treatment in oven at different temperatures proved excellent. All the fungal microflora (yeast and fungal spores) were eliminated by the treatment for 30 min at 80°C. For bacteria, the treatment at 95°C for 30 min is required to reduce the number of bacteria under 102 / g. Schiffman (1992) reported similar results on the use of microwave to reduce microbial content. Olsen (1965) also demostrated the high efficiency of microwave and the total elimination of yeast and fungi.

Finally, an autoclave treatment at 120°C for 15 min was applied. The dry meal was kept in a sealed tube for autoclaving, to avoid direct contact with vapor and the resulting gelatinization. The autoclaving treatment produced best results, because no colony was observed on the plate (< 10 colonia / g DM).

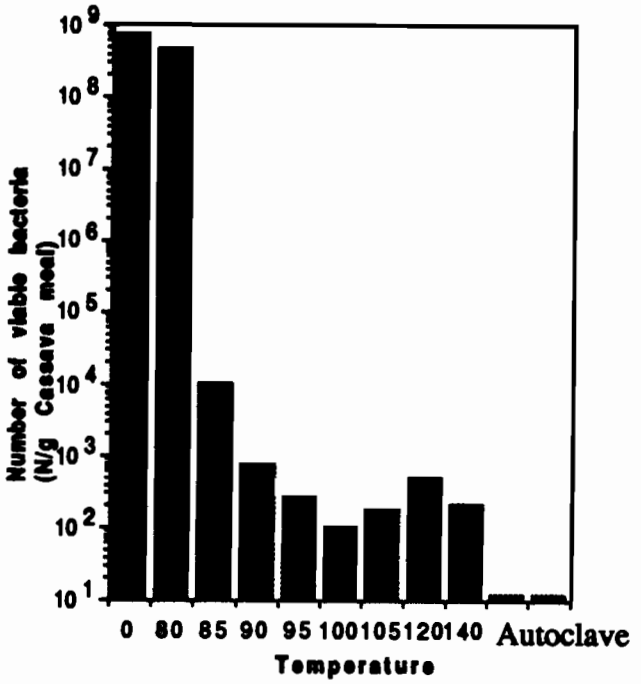

Fig. 3. Effect of temperature on bacterial content of dry cassava meal.

It was concluded that autoclaving treatment of dry cassava meal was sufficient to obtain microbial-free cassava flour for studies on the growth of *Rhizopus* in SSF (Fig. 3).

EFFECT OF HEAT TREATMENTS AND MICROWAVE ON THE GRADE OF GELATINIZATION

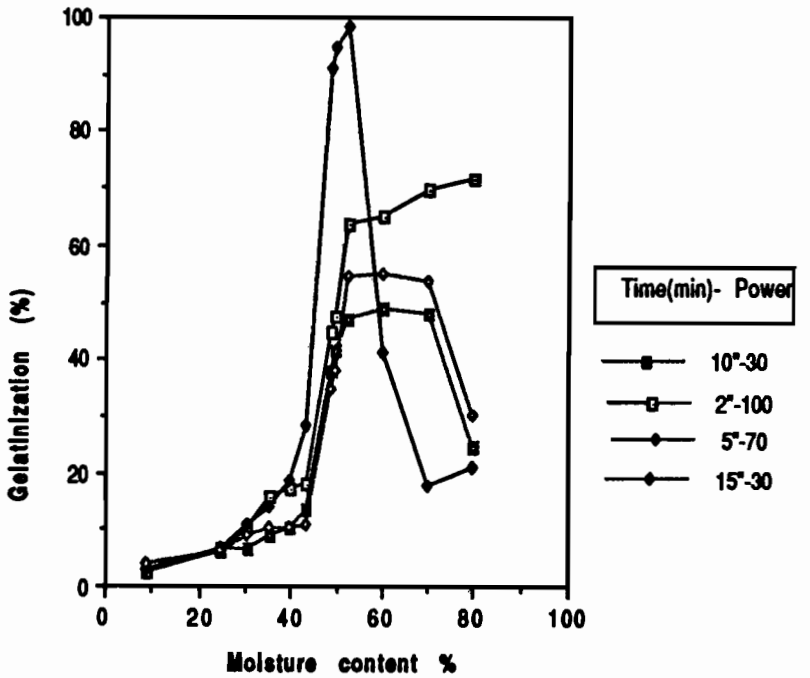

Fig. 4. Effect of micro-waves on gelatinization of cassava meal at various moisture content.

In the case of treatment in a microwave oven, the gelatinization of cassava flour was maximum, when the moisture content was about 50% (Fig. 4). A strange phenomenon of decreased gelatinization was observed for moisture contents higher than 60%, thereby indicating on other kind of starch transformation occurring. However, the aim of the experiment was not to get a high degree of gelatinization of cassava flour. On the contrary, the objective in this study was to find out a procedure of treatment with strong effect on the elimination of contaminant microflora and low

effect on the gelatinization, in view to study the growth of *Rhizopus* in SSF using crude starch. Results obtained by heat treatment were very similar (Fig. 5).

In both cases, it can be observed that, for moisture contents lower than 10% (such as the case of dried meal), both microwave and heat treatment had very low effect on the gelatinization of the starch, even in case of the heating temperature higher than 100°C. This is very important since with such treatment of dry cassava flour, all fungal spore and contaminant microflora could be eliminated, without larger changes in the gelatinization of the starch.

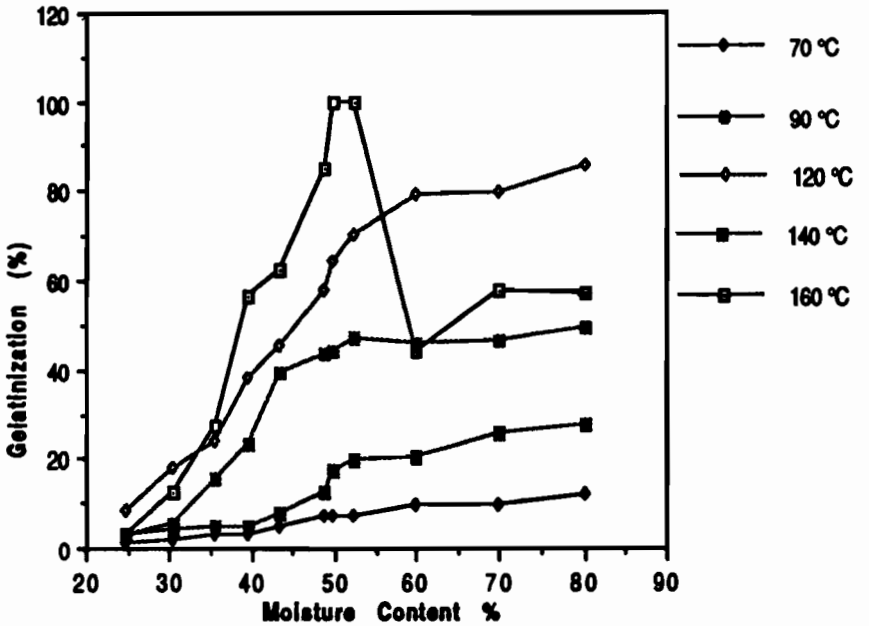

Fig. 5. Gelatinization % at different temperatures for various moisture contents of cassava meals.

Finally, the heat treatment in autoclave in sealed flask was selected for practical reasons at the lab scale. Three kinds of treatment were chosen: a) the dry meal (moisture content< 9%) and gelatinization < 6%, which is considered as crude starch flour, b) the semi-dry treatment (moisture < 30%) and gelatinization = 7%, a very low effect on gelatinization, but allowing prior-humidification of the cassava flour, and c) the wet treatment (moisture content = 40%) with a degree of gelatinization near 30%, similar to the treatment reported by Soccol (1991).

GROWTH OF *RHIZOPUS* ON CASSAVA MEAL OF LOW GELATINIZATION DEGREE

All data on the growth of different strains of *Rhizopus* in the above three categories of cassava flour are not reported here, as was done in various reports on the EEC program. Figs. 6 and 7 show a typical illustration of the respiration kinetics, obtained during the fermentation of *R. oryzae* cultivated in a column containing 150 g of wet product at 50% moisture content, and incubated at 30°C, with an aeration of 100 ml/min. In all these cases, the degree of gelatinization was as low as 7%. A stable RQ (ratio of carbon dioxide production by the oxygen consumption), observed in these studies, indicated a typical vegetative stage and very active growth between 10 and 30 h. On the other hand, Fig. 7 indicates that the exponential phase occurred between 8 and 15 h , was followed by a decrease in growth but *R. oryzae* remained active until 24 h. This simple and direct on-line method allows to determine the specific growth rate of the *Rhizopus* ($\mu = 0.217\ h^{-1}$).

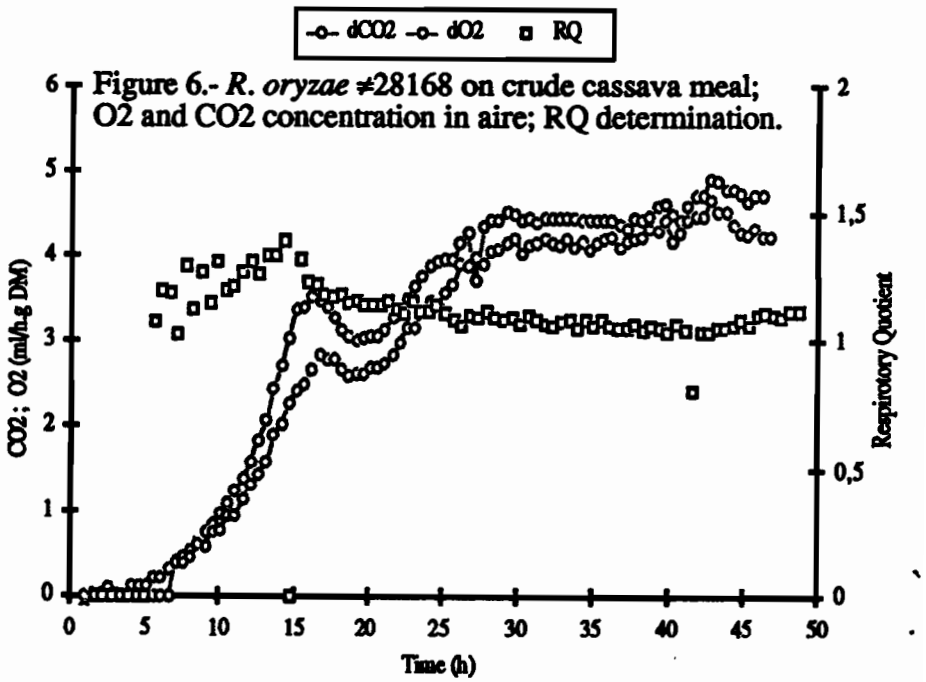

Fig. 6. *R. oryzae* ≠ 28168 on crude cassava meal

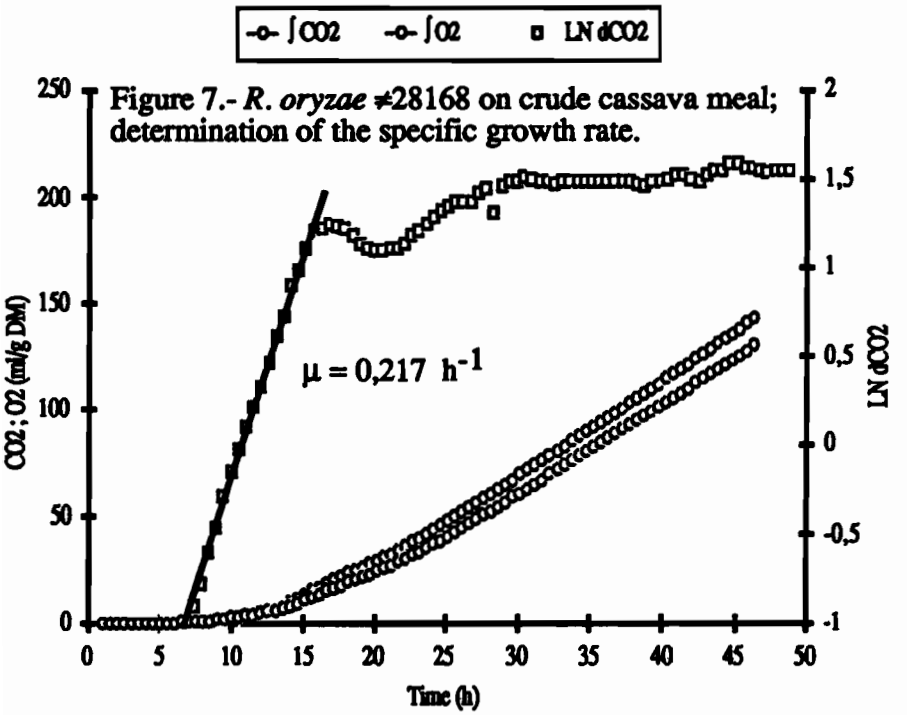

Fig. 7. *R. oryzae* ≠ 28168 on crude cassava meal.

The results on the composition of pure cassava meal and mixed with 50% soybean meal, after autoclave treatment in dry and semi-dry conditions, are presented in Table 3. Very low degree of gelatinization is evident in both cases. With soybean, the mixture is enriched with protein from 11.3 to 20.08%. But the net increase is comparable and in the case of pure cassava meal enrichment, the protein content increased from 4.15 to 14%.

Table 3. Comparison of the growth of *Rhizopus oryzae* on cassava meals treated in autoclave in dry (7% moisture) or semi-dry (30% moisture) for 20 min in sealed containers.

	DRY MEAL	SEMI DRY MEAL
Gelatinization,%	6.31	7.11
μ = Growth rate, 1/h		
Pure cassava	0.056	0.217
Cassava + soybean		
Final protein content		
Pure cassava	4.15	14
Cassava/soybean	11.31	20.08

CONCLUSION

It was concluded that *Rhizopus oryzae* is capable of degrading crude starch, after a light heat treatment that eliminated the contaminant microflora. It was observed that the treatment in semi-dry condition, i.e., at 30% moisture content and 7% gelatinization, improved significantly the specific growth rate and the respiratory activity of *Rhizopus*. However, the mixture with soybean meal did not allow better protein yields. It is necessary to investigate further the screening of *Rhizopus* sp. so that knowledge on the degradation of crude starch by specific strains could be improved. The production and biosynthesis of glucoamylase is under investigation to verify whether the concentration of enzyme is higher, when *Rhizopus* is grown on crude cassava, than on gelatinized starch.

ACKNOWLEDGEMENTS

This work was supported by an EEC project STD3, and by ORSTOM in the frame of the ORSTOM/Univalle co-operative programme.

REFERENCES

Mitchell, D.A., Doelle, H.W. and Greenfield, P.F. 1988. Agar plate growth studies of *Rhizopus oligosporus* and *Aspergillus oryzae* to determine their suitability for solid state fermentation. *Appl. Microbiol. Biotechnol.* 28: 598-602.

Olsen, C.M. 1965. Microwave inhibit bread mold. *Food Eng.* 37: 51-

Raimbault, M. 1981. Fermentation en milieu solide: Croissance de champignons filamenteux sur substrats amylacée. *Thesis Doctorat* Toulouse, Doc Orstom No. 172, Paris.

Raimbault, M. and Alazard, D. 1980. Culture method to study fungal growth in solid fermentation. *Eur. J. Appl. Microbiol. Biotechnol.* 9: 199-209.

Saucedo-Castañeda, G., Trejo-Hernandez, M.R., Lonsane, B.K., Navarro, J.M., Roussos, S., Dufour, D. and Raimbault, M. 1994. On-line automated monitoring and control systems for CO_2 and O_2 in aerobic and anaerobic solid state fermentations. *Process Biochem.* 29: 13-24.

Schiffman, R.E. 1992. Microwave processing in the U.S. food industry. *Food Technol.* 46: 50-52.

Soccol, C.R., Marin, B., Raimbault, M. and Lebeault, J.M. 1994. Breeding and growth of *Rhizopus* in raw cassava by solid state fermentation. *Appl. Microbiol. Biotechnol.* 41: 330-336.

Soccol, C., Rodriguez, L.J., Marin, B., Roussos, S. and Raimbault, M. 1993. Growth kinetics of *Rhizopus arrhizus* in solid state fermentation of treated cassava. *Biotechnol. Tech.* 7: 563-568.

Soccol, C.R., Iloki, I., Marin, M. and Raimbault, M. 1994. Comparative production of ∝–amylase, glucoamylase and protein enrichment of raw and cooked cassava by *Rhizopus* strains in submerged and solid state fermentations. *J. Food Sci. Technol.* 31: 320-323.

Wotton, M., Weedon, D. and Munck, N. 1971. A rapid method for estimation of starch gelatinization in processed foods. *Food Technol. Austr.* 23: 612-614.

Raimbault Maurice, Ramirez Toro C. (1997).

Growth of Rhizopus sp. on ungelatinized cassava flour in solid state fermentation for protein enrichment.

In : Roussos Sevastianos (ed.), Lonsane B.K. (ed.), Raimbault Maurice (ed.), Viniegra-Gonzalez G. (ed.) Advances in solid state fermentation : proceedings of the 2nd international symposium on solid state fermentation. Dordrecht : Kluwer, 169-181.

FMS-95 : Solid State Fermentation : International Symposium, 2., Montpellier (FRA), 1997/02/27-28. ISBN 0-7923-4732-3