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SCANNING ELECTRON MICROSCOPY OF THE DEVELOPMENT OF *RHIZOPUS ARRHIZUS* ON RAW CASSAVA BY SOLID STATE FERMENTATION

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MICROSCOPIA ELECTRONICA DE BARRIDO DEL DESARROLLO DE *RHIZOPUS ARRHIZUS* SOBRE YUCA CRUDA EN FERMENTACION SOLIDA

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SUMMARY

Some morphological aspects of the growth of *Rhizopus arrhizus* on raw cassava were studied. The interactions between substrate and mycelium were observed during different phases of development, such as aspect of the flour, spore morphology and germination, fungal growth on the granular raw cassava, and formation of sporangia, including maturation and release of the spores. Protein content after fungal growth was of 10.8%, which indicated an enrichment of the substrate of about 9%.

Key words: Raw cassava, solid state fermentation, *Rhizopus arrhizus*, scanning electron microscopy.

RESUMEN

Se estudió el crecimiento de una cepa de *Rhizopus arrhizus* sobre yuca granular cruda, con la ayuda de la microscopía electrónica de barrido. Se describen las

diferentes etapas de crecimiento, así como la relación substrato-microorganismo, presentando observaciones microscópicas de la germinación, desarrollo del tubo germinativo, crecimiento micelial y la colonización completa de la superficie en 40 horas. Las observaciones del micelio, después de 40 horas de cultivo, permitieron reconocer algunos aspectos de la reproducción asexual y la liberación de esporangiosporas de *R. arrhizus*. Al final de la fermentación, el contenido de proteína fue de 10.8%, lo cual representó un enriquecimiento promedio del substrato de 9%, constituído esencialmente por el micelio del hongo. Estas observaciones demuestran la capacidad de dicho hongo filamentoso para crecer sobre yuca cruda, además de nuevas perspectivas para el aprovechamiento de esta materia prima tropical.

Palabras clave: Yuca cruda, fermentación sólida, *Rhizopus arrhizus*, microscopía electrónica de barrido.

INTRODUCTION

The genus *Rhizopus* comprises a group of widespread species belonging to the order Mucorales (Samson and Reenen-Hoesktra, 1988). Some of them are very important for the food industry, involving solid substrate fermentation processes which have been used by man for many centuries, especially in the Asian countries, such as China, Korea, Japan, Indonesia, Malaysia, and Singapore (Hesseltine, 1965; Raimbault, 1980; Soccol, 1986). These fungi are able to increase the food digestibility, to improve the protein content (Soccol, 1992; Soccol *et al.*, 1992; Beuchat, 1987; Daubresse *et al.*, 1987), to disturb the synthesis of aflatoxins (Zhu *et al.*, 1989), to produce anticarcinogenic molecules (Ko, 1988) and some very effective antibiotics against a great number of bacteria (Wang *et al.*, 1969), and to detoxify cassava from the cyanogenic glycosides (Padmaja and Alagopal, 1985).

In previous studies (Soccol, 1992; Soccol *et al.*, 1992), it was found that some strains of *Rhizopus* were able to grow successfully on raw cassava by solid state fermentation (SSF). In this paper, morphological aspects from this process were studied by scanning electron microscopy, as well as some biochemical parameters (protein and carbohydrate contents).

MATERIALS AND METHODS

Preparation of solid medium

The solid medium used for fermentation was 10 g of dry raw cassava, with a

granulometry of 0.8 - 2 mm. The biological material was moistened at 50% saturation with a mineral solution, whose composition was: 4.7% KH_2PO_4, 9.4% $(NH_4)_2SO_4$ and 2.3% urea. Initially, this solution was buffered at pH 5.8 with ammonia before addition of spores. Inocula were prepared with 2×10^7 spores of *Rhizopus arrhizus* Fisher (ATCC-34612) per g dry cassava. Homogenization was performed by distributing homogeneously the mineral solution and spores in the cassava biomass, with a mixing shaker during 2-3 min. Thereafter, 20 g of the inoculated material were placed into polypropylene petri dishes (13 cm diameter).

Cultivation of the fungus

Cultivation was conducted at 35°C during 60 h, in a glass incubator with an adequate aperture for gaseous exchange (Fig. 1). The petri dishes were placed on a perforated porcelain support. The lower part of the incubator had distilled water to permit air saturation.

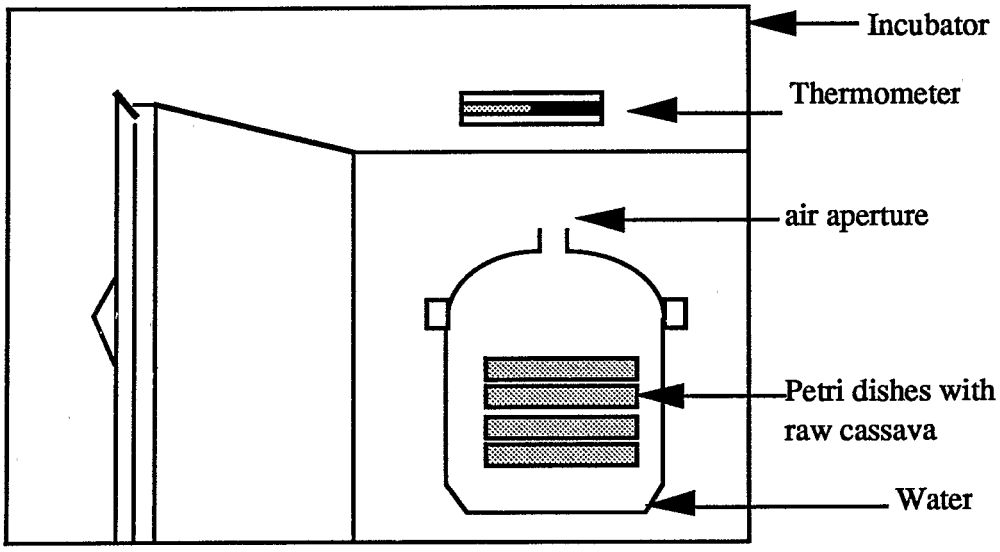


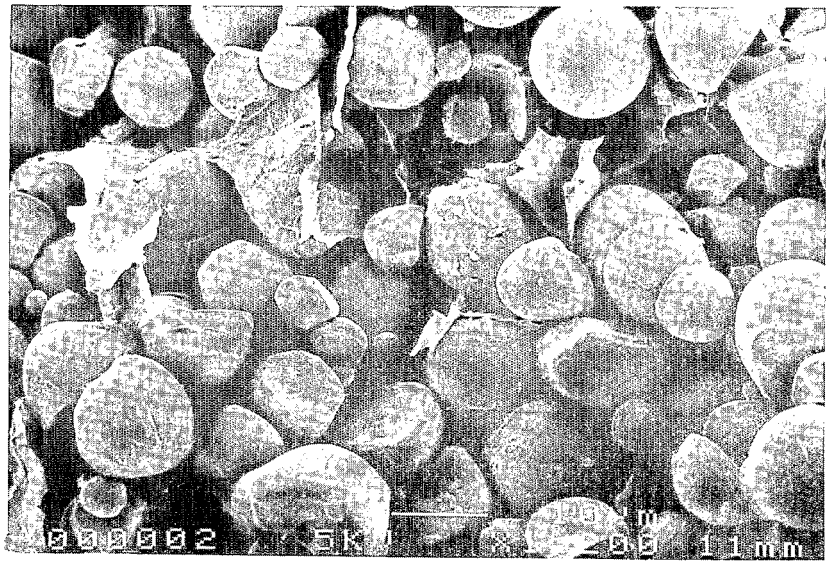
Fig. 1. Incubation of *Rhizopus arrhizus* growing on raw cassava in petri dishes in the presence of humidified air.

Sampling and scanning electron microscopy (SEM)
Growth was analyzed by sampling at different times during cultivation. In each case, protein content was determined by the Lowry method (Lowry *et al.*, 1951), and total glucosides were estimated by the anthron method (Dubois *et al.*, 1956). Samples were immersed during 12 h in a buffered solution of glutaraldehyde in order to avoid any modification of the dehydrated cellular structures and to obtain a homogeneous metallization process. The composition of the fixing solution was as follows: 1 ml 25% glutaraldehyde, 4 ml cacodylate buffer (prepared with 16 g L^{-1} cacodylic acid, pH 7.5), and 1 ml distilled water. After fixation, the samples were rinsed with distilled water, and then gradually dehydrated with the following concentrations of ethanol: 10%, 20%, 40%, 60%, 80%, 95%, and 100%. Each step was carried out twice during 30 min at every concentration. The samples were then cryogenicly dried at critical point with liquid CO_2. The completely dry samples were coated with gold/palladium using Polaron model E 5000 (Saucedo-Castañeda, 1991). Micrograph analysis was performed with a scanning electron microscopic apparatus JEOL, model J. S. M-35.

RESULTS AND DISCUSSION

Morphological analysis
Substrate and spores. Cassava flour is formed by smooth and spherical amylaceous granules of heterogeneous size (5-15 µm). In certain cases, some plant debris could be evidenced (Fig. 2). When inoculated, at time 0, the typical morphology of sporangiospores (7-10 µm in size) could be observed having irregular shape with a striped surface and grooves along the longitudinal axis. They were usually present in the free intergranular space of the cassava flour (Fig. 3).

Germination. The germ tube began to be observed after 8-12 h of incubation (Figs. 4-5). During this period, the spores were swelling and their shape became globular (13 µm diameter) with the surface devoid of grooves. A similar phenomenon has been observed using liquid medium after only 3 h of culture. Most spores germinated after 20 h. The germ tube had a mean length of 15 µm, and 3-5 µm in diameter. Most hyphae began to grow on the granules of raw cassava (Fig. 6).



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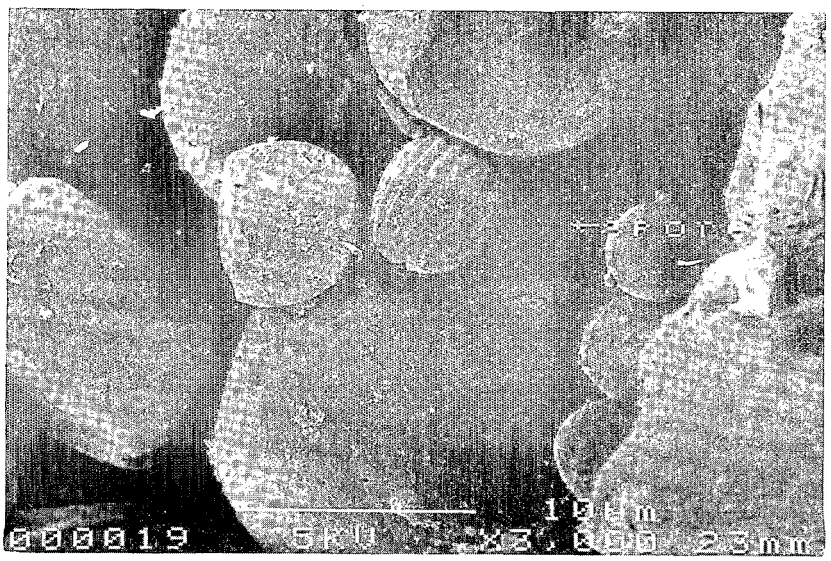

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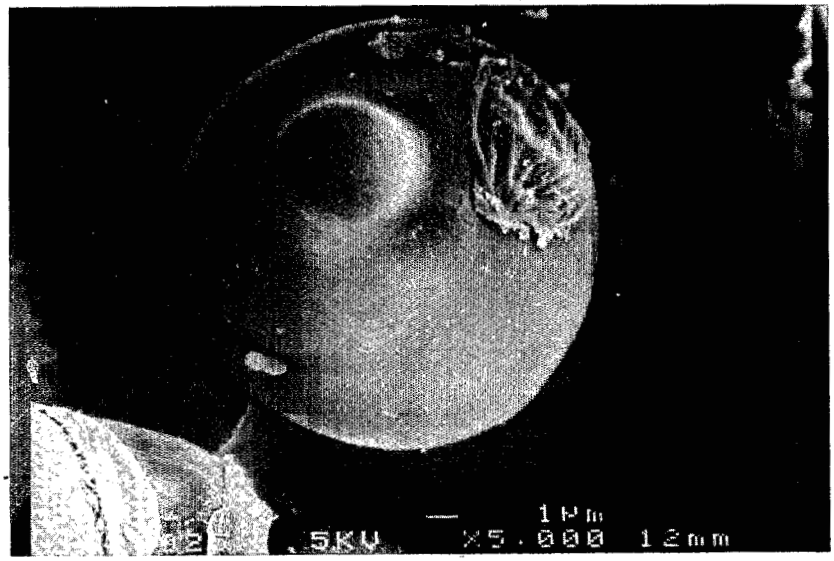
Figs. 2-3.- 2: SEM of the granules of raw cassava flour. 3: SEM of sporangiospores of *Rhizopus arrhizus* scattered on raw cassava.



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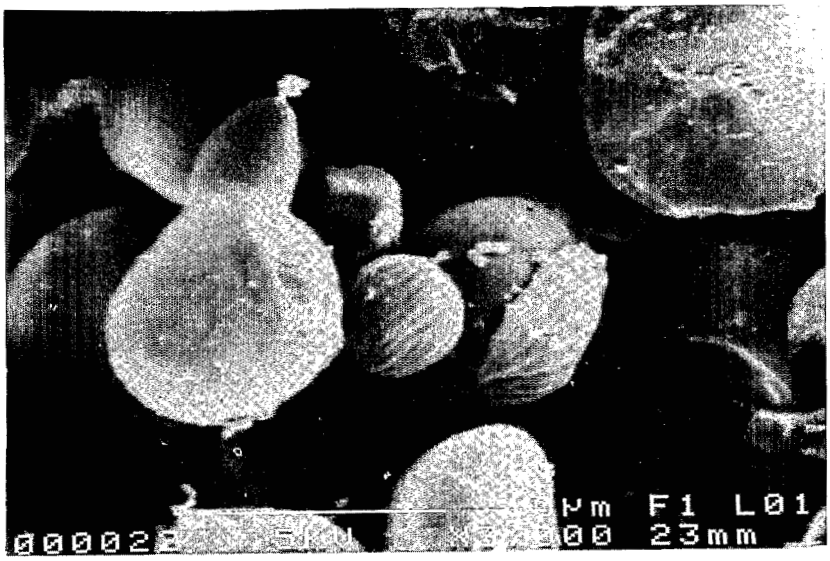

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Figs. 4-5.- SEM of sporangiospores of *R. arrhizus*. 4: Swelling. 5: Germination.

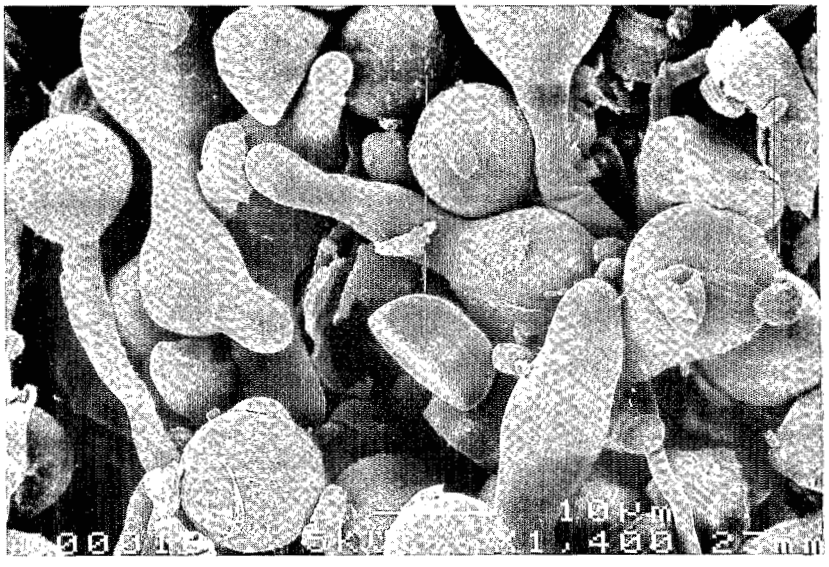
Substrate colonization. A mycelial network was developed on the granules of raw cassava after 30 h of culture, indicating an exponential growth stage. There were not granules of cassava free of mycelium at this stage (Fig. 7). After 40 h of culture, the loss of substrate was significantly correlated with a further growth of fungal filamentous hyphae (Figs. 8-9). This can explain the high protein content of the final product found in the fermenter. Some hyphae initiated the differentiation of typical sporangia (Fig. 10).

Spore discharge. Maturation of sporangia usually took place after 48 h of culture. This phase was completely achieved after 56 h of culture (Figs. 11-12), and at its end the sporangia had a diameter of 90-120 µm. Thereafter, the sporangial wall was broken into many small fragments allowing the release of the spores (Fig. 11). Each sporangia contained about 3,000 spores. After 60 h of culture, the mycelial biomass became very dense and blackish due to the intensive liberation of spores by the sporangia (Fig. 13).

Biochemical analysis

The different biochemical parameters on the growth of *Rhizopus arrhizus* on raw cassava are shown in Fig. 14, *i.e.* the kinetics of protein content, total carbohydrate consumption, and the decrease of pH in the medium. The protein content of the medium increased linearly after 20 h of culture up to the end of the fermentation. After 56 h of culture, this content was estimated to be 18.8% (with respect to the initial dry weight). A yield of 39% for proteins could be calculated from the consumption of carbohydrates. On the other hand, the pH of the medium decreased during the fermentation process in the conditions used. As the buffering capacity of the medium was not important, such a linear decrease could be related to the fungal synthesis of an acid metabolite. Further studies have identified this molecule as fumaric acid (Soccol, 1992).

The use of raw cassava without any thermal treatment, as described in this study, is a new alternative for using such byproduct.



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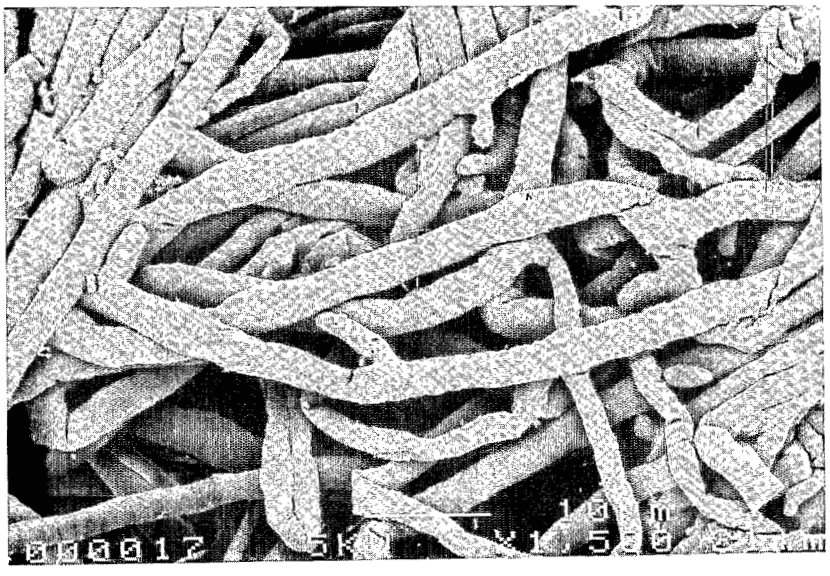

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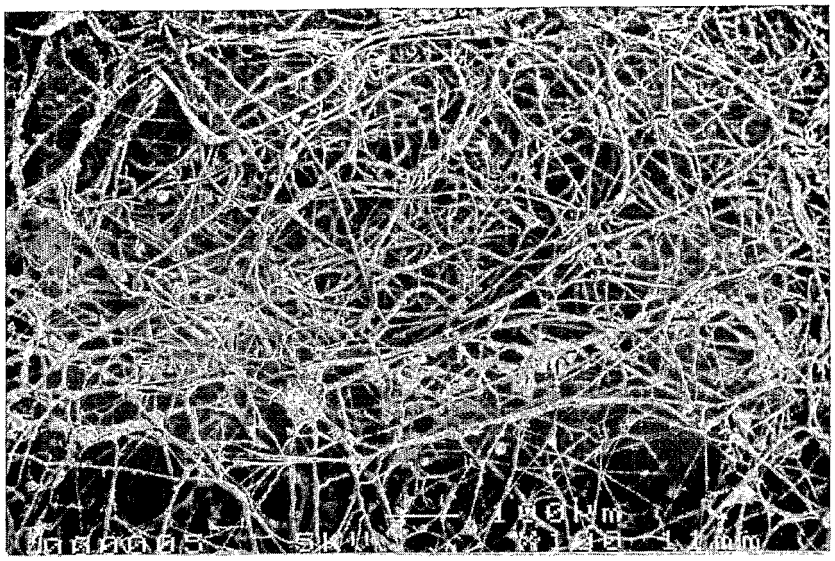
Figs. 6-7.- 6: SEM of germinated sporangiospores of *Rhizopus arrhizus*. 7: SEM of the mycelial growth on raw cassava.



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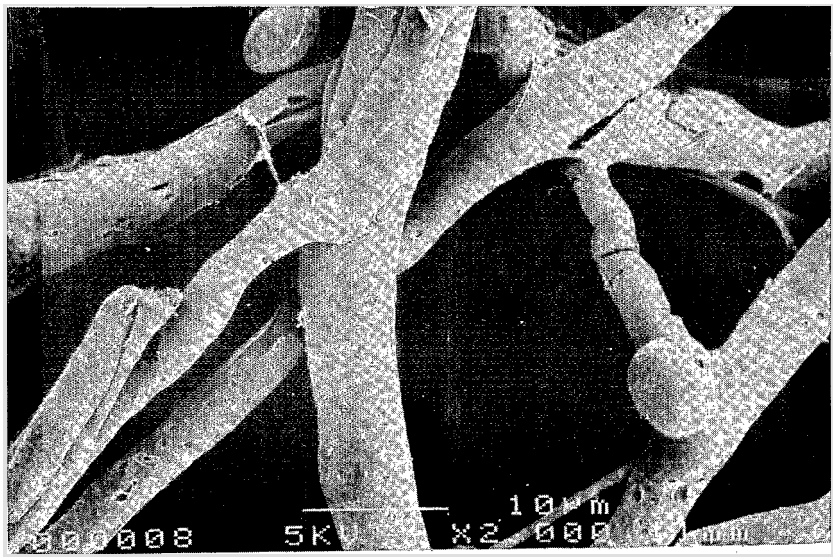

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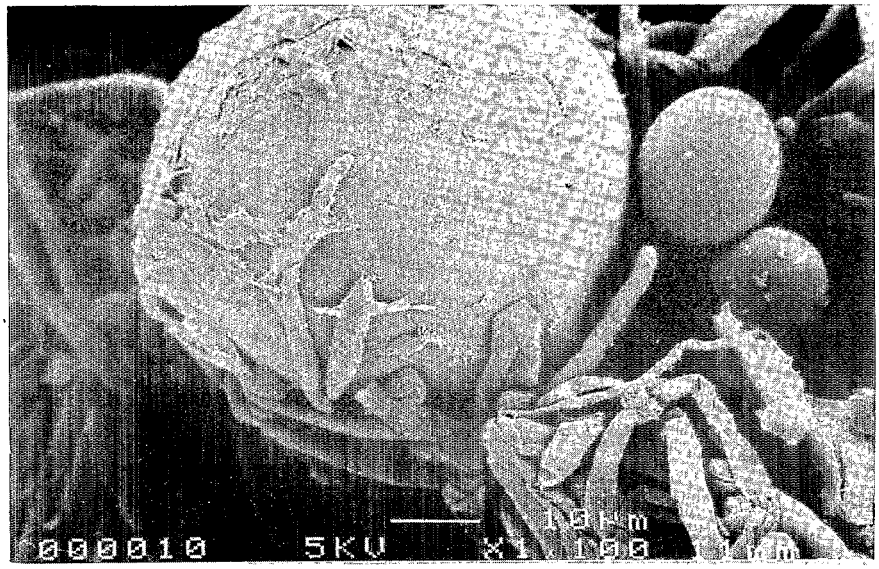
Figs. 8-9.- 8: SEM of the mycelial biomass of *Rhizopus arrhizus* encapsulating cassava granules. 9: SEM of the beginning of sporangia differentiation.



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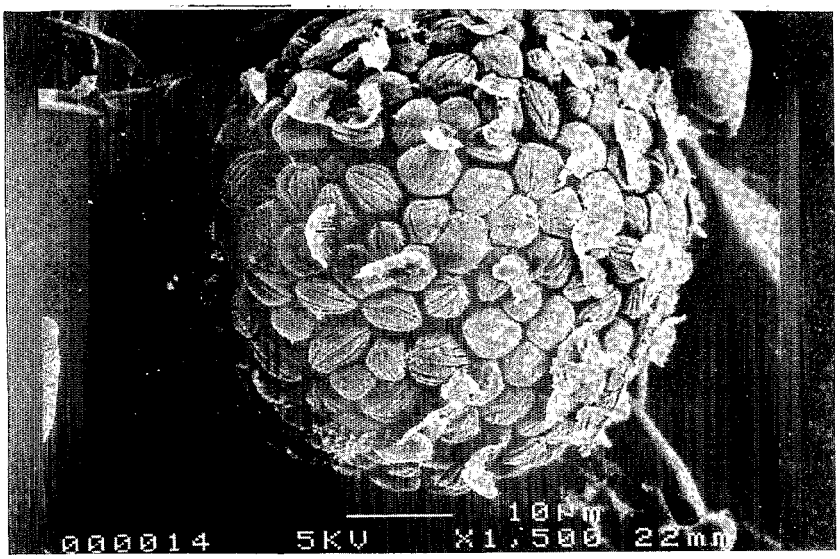

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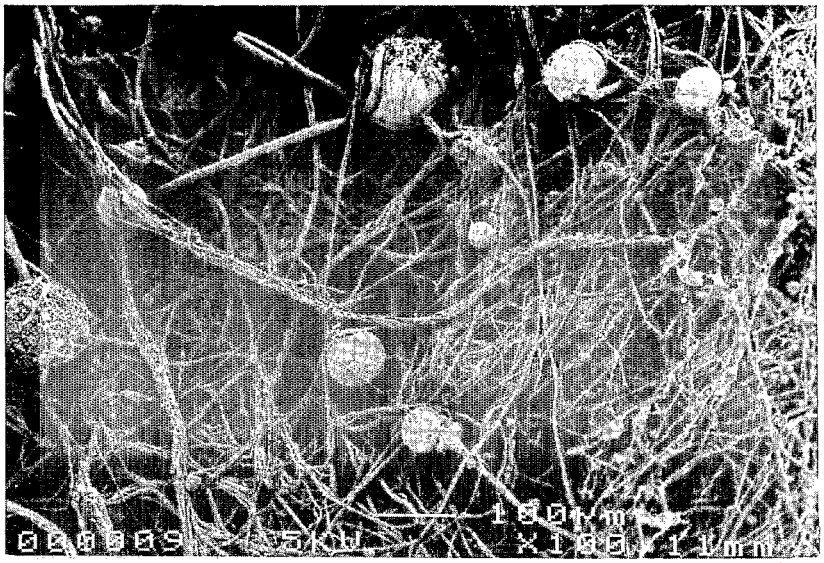
Figs. 10-11.- 10: SEM of the maturation of sporangia in *Rhizopus arrhizus*. 11: SEM of a mature sporangia capable to release the spores.



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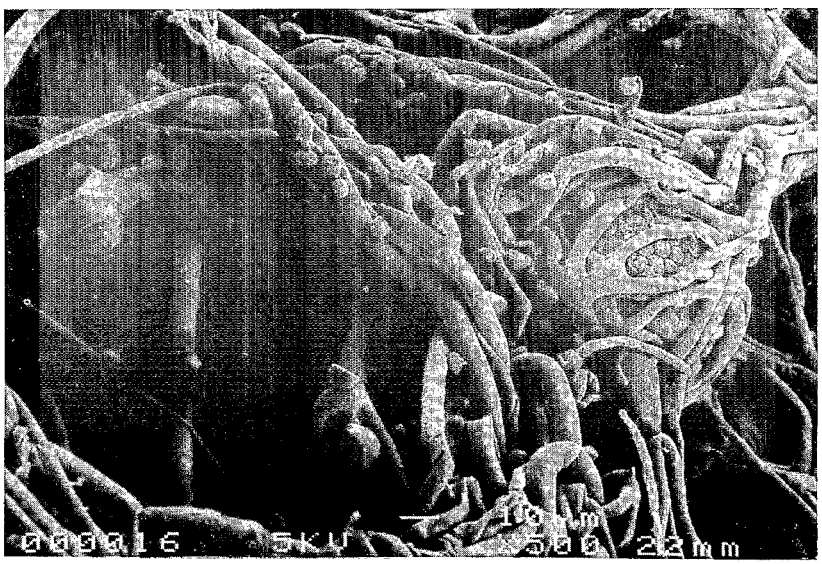

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Figs. 12-13. SEM of the sporangia and abundant released sporangiospores of *Rhizopus arrhizus*.

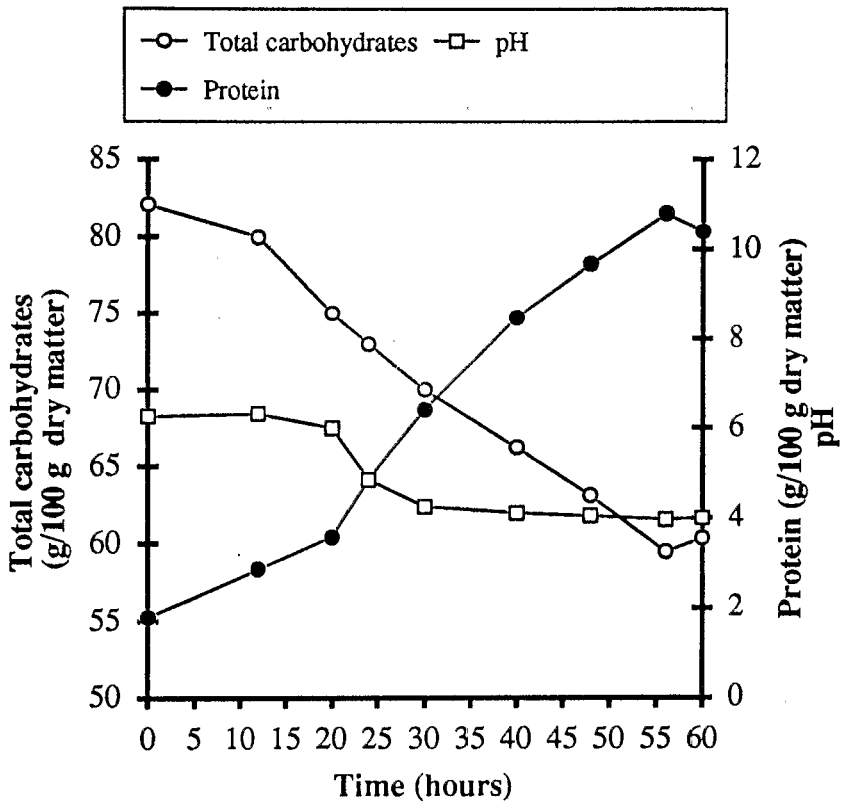

Fig. 14. Evolution of protein and total carbohydrate contents, as well as pH of the medium, during the growth of *Rhizopus arrhizus* on raw cassava.

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