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Potential of Using a Single Fermenter for Biomass Build-Up, Starch Hydrolysis, and Ethanol Production

Solid State Fermentation System Involving *Schwanniomyces castellii*

G. SAUCEDO-CASTAÑEDA,*[1] B. K. LONSANE,[2] J. M. NAVARRO,[3] S. ROUSSOS,[4] AND M. RAIMBAULT[4]

[1]*Department of Biotechnology, Autonomous Metropolitan University, Iztapalapa Campus, A. P. 55-535, C.P. 9340, Mexico City, Mexico;* [2]*Fermentation Technology and Bioengineering Discipline, Central Food Technological Research Institute, Mysore 570 013, India;* [3]*Microbiologie Industrielle GBSA, Université Montpellier II, Place E. Bataillon, 34095 Montpellier, France; and* [4]*ORSTOM, Centre de Montpellier, Biotechnology Unit, 911, Avenue Agropolis, B.P. 5045, 34032 Montpellier Cedex 1, France*

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ABSTRACT

Data on conversion of starch on biomass and ethanol by *Schwanniomyces castellii* in an aerobic-anaerobic solid state fermentation is reported. *Schwanniomyces castellii* grew exponentially in the aerobic phase (12 h) and simultaneously hydrolyzed nearly half (55%) of the starch initially present. The accumulation of glucose increased up to 12 h, whereas maltose was nearly absent beyond 7 h. Shift of metabolism from oxidative to fermentative pattern was observed about 10 h as a result of the build-up of CO_2 level and faster utilization of O_2. The ethanol production in the anaerobic phase reached the level of 89.3 mg ethanol/g initial dry matter by the end of 30 h. A total of 92.9% of the starch is utilized during the fermentation. The overall ethanol

*Author to whom all correspondence and reprint requests should be addressed.

conversion yields are 57.8% of the theoretical value, whereas in the anaerobic phase it was found to be 94.4%. The cell shape, its morphology, and the type of attachment to the solid support were found to be similar in aerobic and anaerobic phases of fermentation. Data given in this work indicate the feasibility of using one single fermenter for aerobic growth to generate inoculum as well as to simultaneously hydrolyze the starch and subsequent anaerobic fermentation to produce ethanol.

Index Entries: *Schwanniomyces castellii;* ethanol production; solid state fermentation; column fermenter; inert solid support; pith bagasse; biomass build-up; starch hydrolysis; growth characteristics; aerobic and anaerobic phases.

INTRODUCTION

Schwanniomyces castellii is an interesting alternative to conventional *Saccharomyces cerevisiae* for production of ethanol from starchy substrates (*1–4*). It offers advantages of practical and economic significance, such as elimination of prior hydrolysis of starch, homofermentative nature of the culture (*3*), a conversion efficiency of greater than 95% (*3*), and avoidance of the use of mixed or associative fermentations that otherwise is essential in case of starchy substrates (*5*). Its lower tolerance to ethanol (*2*) could be overcome by continuous stripping of ethanol by means of inert gas circulation, the condensate could be used for alcoholic beverages making without redistillation (*6*). Solid state fermentation (SSF) systems have been proposed as an alternative to submerged fermentation for alcohol production, sugar crops have been directly fermented at farm scale (*7*). Economics of solid-phase fermentation for ethanol production has been reported elsewhere (*6,8*). Our efforts revealed that SSF systems for ethanol production could be used instead of liquid state fermentation by using an amylolytic fermenting yeast (*4,9*). Scale-up of this process has been studied by maintaining heat and water balances in culture media in a previous paper (*5*). The process consists of aerobic growth of the culture for biomass build-up as well as production of enzymes for starch hydrolysis, and conversion of glucose thus formed into ethanol in the subsequent anaerobic fermentation with continuous stripping of ethanol from the medium by recirculation of exhaust gases from the fermenter (*5*). It will also eliminate the need for preparing large inoculum for industrial fermentation. The unit operation has been characterized as problematic by Bank (*10*) in the scale-up of the processes.

The present communication reports the patterns of biomass build-up, starch hydrolysis, glucose concentration, and ethanol production in a single solid state column fermenter. In addition, the morphology of the cells and the type of cell attachment to the solid support particles in aerobic and anaerobic phases of the fermentation are also reported. The

information on these aspects is useful for process optimization and also for developing efficient method for estimation of growth. No such data on these aspects were available earlier for SSF system though few electron microphotograph of *Schwanniomyces castellii* on different solid starchy substrates have been published to indicate that the growth increases with time in aerobic SSF process for protein enrichment (*11*).

MATERIALS AND METHODS

Microorganism

Schwanniomyces castellii CBS 2863, obtained from Centraal Bureau Voor Schmmelcultures (Delf, Holland), was maintained on agar slants (*12*) at 4°C by subculturing every month. For inoculum preparation, it was grown in the medium of Oteng-Gyang (*13*) on shaker at 28–30°C for 24 h to the cell density of 5×10^8 cells/mL.

Cultural Technique

Pith bagasse is a byproduct of sugar cane industry, it was used as inert solid support for absorbing a liquid culture medium (*see below*). Pith bagasse was sieved to obtained particle size of 0.3–0.8 mm and transferred to 0.3 mm SS sieve for thorough washing (twice) using 10 times (w/w) deionized water at each stage. It was sterilized at 121°C for 30 min in the same sieve at 5 cm depth. Autoclaved bagasse was transferred to plastic trays at the depth of 2 cm for drying in the oven at 60°C for about 30 h. In this way, bagasse can be stored without deterioration until its utilization.

Basal liquid nutrient medium (pH 3.5) contained (g/L): soluble starch (Prolabo) 100, peptone 1, urea 1.3, yeast extract 1, KH_2PO_4 5, NaCl 1, $MgSO_4 \cdot 7H_2O$ 2. The medium was sterilized at 121°C for 15 min, cooled to about 30°C, mixed with 2 mL filter sterilized vitamin-mineral solution (*14*), adjusted to pH 5.7 inoculated at 10% (v/v) level. The inoculated medium was mixed with the processed pith bagasse at the ratio of bagasse to liquid medium of 14:86 (w/v). Aseptic conditions were maintained only up to the mixing of the support with inoculated liquid medium. Initial moisture content of the medium thus prepared was about 77%.

Fermentation was carried out in column fermenter of the size of 4 cm dia × 20 cm length. Each column was charged with 60 g of the inoculated moist solid medium and incubated at 30°C in a water bath (Fig. 1). The other design features of the fermenter are as reported elsewhere (*15*). The fermenters were supplied with humidified air in the first 12 h of fermentation, at a air flow rate of 1.4 mL/min per g wet material, but the air supply was switched off subsequently. The CO_2 produced was allowed to escape in the atmosphere after bubbling through water in order to mainain anaerobic conditions (Fig. 1), except in case of one of the columns, designed as F, which was used for gas analysis (Fig. 2).

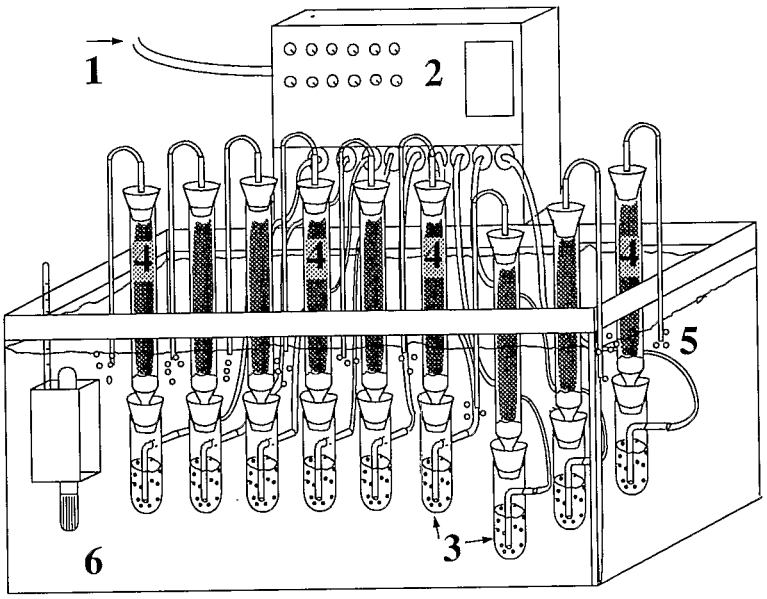

Fig. 1. Diagram of the solid state fermentation system for ethanol production. 1: Air supply; 2: Aeration valves panel; 3: air humidifier; 4: column fermenter; 5: bubbling tube; 6: water bath at controlled temperature.

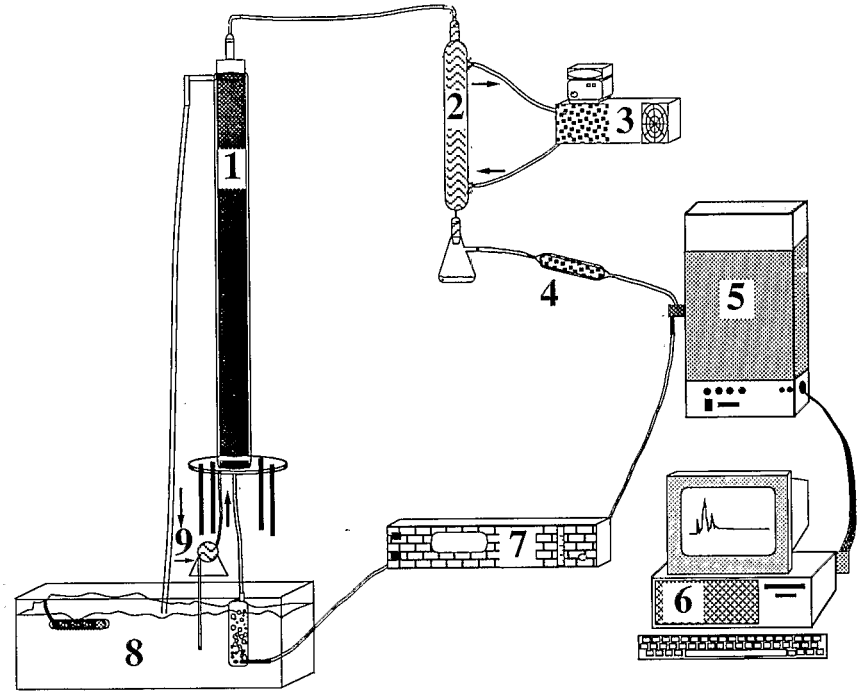

Fig. 2. Experimental set-up for gaz analysis in solid state fermentation system for ethanol production. 1: Fermenter "F"; 2: condenser; 3: chilled water batch; 4: silica gel column; 5: gas chromatograph; 6: computer; 7: air pump; 8: temperature controlled water bath.

The fermentation was continued up to 30 h in the column F with stripping of ethanol formed and recirculation of exhaust gases (Fig. 2). The column F is equipped with water jacket, humidification of air supply, water trap before the entry of humidified, and heated air in the fermenter, console for stripping of ethanol from fermenting medium, on line monitoring for CO_2 and O_2 and the membrane pump for recirculation of exhaust air. In case of combined aerobic and anaerobic fermentation, the total air supply to the fermenter F was 400 mL, which was recirculated by means of membrane pump at a rate of 20 mL/min. The exhaust air during the aerobic and anaerobic phases of fermentation was passed through a laboratory condenser which was cooled by chilled water from the bath maintained at 2°C. It thus continuously stripped ethanol during anaerobic phase. The exhaust gas was then dried by passing through a silica gel column for gas analysis and recirculation through the fermenter.

The combined aerobic and anaerobic phases of fermentation as well as only aerobic fermentation throughout the period of 30 h were also carried out in six segmented column fermenter of 370 g moist solid medium capacity (1L total capacity), similar to fermenter F (Fig. 2). In these cases, the aeration rate was either at 0.8 or 1.5 mL/min/g wet material. The detailed description of the fermenters, their operation strategy and other related fermentation aspects have been described elsewhere (*5,9*).

Analytical Aspects

Fresh samples (5g) were diluted with 45 mL of distilled water. The concentration of various carbohydrates and ethanol in the fermenting solids were determined by HPLC using the procedure described elsewhere (*16*). The CO_2 and O_2 concentrations in the exhaust air were measured by gas chromatography provided with an automatic injector that was coupled to personal computer through the integration program. Estimations were also made of moisture and dry cell biomass. Details of these methodologies have been reported elsewhere (*5,9*).

Scanning Electron Microscopy

The processed pith bagasse in which the culture is grown aerobically (12 h), and the material which has passed through aerobic and anaerobic phases of growth (30 h) were subjected to scanning electron microscopy (Model S360, Cambridge Instruments, Cambridge, UK) to study the growth characteristics. The preparation of the sample involved four steps, i.e., fixation to prevent deformation of the structure, complete dehydration of the material, total removal of ethanol (dehydrating agent) by application of CO_2 at critical point and homogeneous gold metallization. The methodology used was similar to that reported by Blaha and Paris (*17*).

The fixation of the material was done using two different methods. In one case, the samples were fixed by formaldehyde-acetic acid-ethanol solution (10:10:85; the strength of formaldehyde used was 40%). The fixation solution was allowed to react with the material for 12 h under vacuum so as to completely eliminate air bubbles from the moist solid medium and also to facilitate the reaction. The fixed material was passed through alcohol baths in succession and by increasing concentrations of ethanol, i.e., twice through 95 and 100% ethanol, for dehydration that was allowed to proceed for 12 h under vacuum in each ethanol bath. Samples were then conserved in absolute ethanol until they were subjected twice to CO_2 at critical point (Balzers, Liechtenstein) to completely eliminate ethanol. Finally, the material was subjected to metallization by a fine gold layer of 200–250 Angstroms (Model E5000, Polaron, UK).

Another fixation technique involves the use of 1 mL of 25% glutaraldehyde (Sigma), 4 mL of cacodylate buffer (Sigma; 16 g/L, pH 7.5), and 1 mL of distilled water. For fixation, the sample was washed twice gently with fixation solution at 1 h intervals under vacuum, and then it was allowed to react for 12 h under vacuum. Samples were washed twice gently with cacodylate buffer (without glutaraldehyde) at 1 h interval under vacuum. The dehydration was achieved by increasing ethanol concentrations to 10, 20, 40, 60, 80, 95, and 100%. Dehydration was allowed to proceed for 12 h under vacuum in each ethanol bath. The samples were conserved in absolute ethanol until application of CO_2 at critical point and gold metallization, as described earlier.

RESULTS AND DISCUSSION

Biomass Build-up and Starch Hydrolysis

The data on the biomass build-up and starch hydrolysis (Fig. 3) in the aerobic and anaerobic phases of fermentation in a single fermenter indicate active growth of the culture up to 12 h, resulting in 41.7 mg dry cells/g IDM. A slight decrease in biomass concentration during the anaerobic phase resulted in a negative biomass yield (Table 1). Nearly half (55%) of the starch was hydrolyzed during the aerobic phase (12 h). This confers an additional benefit for carrying out both the fermentation phases in a single fermenter as it saves considerable time that otherwise would be required if these phases are performed separately. A lower rate of starch hydrolysis was observed beyond 15 h in the subsequent anaerobic phase. The accumulation of glucose in the medium increased progressively up to 12 h in contrast to that of maltose only up to 7 h (Fig. 3). The maltose concentration in the medium was negligible or zero in the subsequent anaerobic phase.

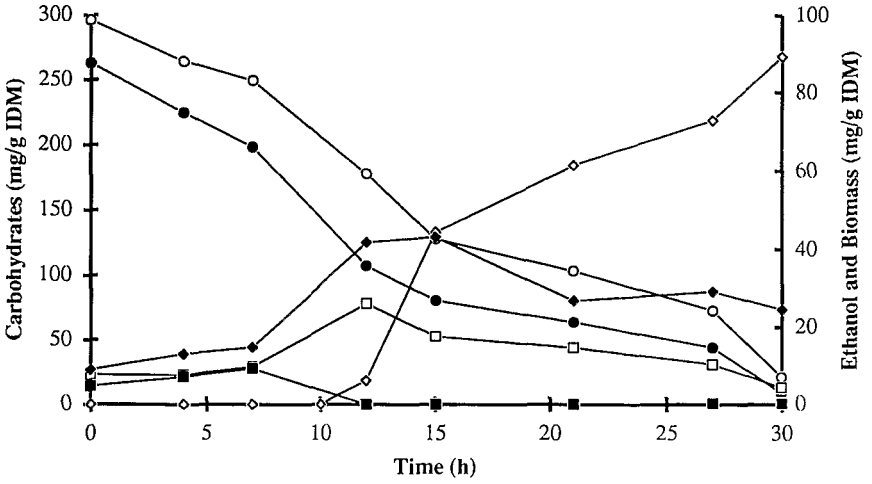

Fig. 3. Patterns of total carbohydrates, starch, glucose, maltose, biomass, and ethanol concentrations during aerobic and anaerobic phases in a single column fermenter of 60 g moist medium capacity. ○: total carbohydrates; ●: starch; □ glucose; ■: maltose; ◇: ethanol and ◆: biomass.

Table 1
Data on Conversion of Starch into Biomass and Ethanol by *Schwanniomyces castellii* in a Solid State Fermentation System*

Fermentation	Attribute	Value, g	Yield
Overall	Initial carbohydrates present	29.6	100.0[a]
	Carbohydrates consumed	27.5	92.9[a]
	Biomass produced	1.6	0.06[b]
	Ethanol formed	8.9	57.8[c]
Aerobic phase	Carbohydrates consumed	11.8	40.0[a]
	Biomass produced	3.4	0.29[b]
	Ethanol formed in transition phase	0.6	9.1[c]
Anaerobic phase	Carbohydrates consumed	15.7	52.9[a]
	Biomass produced	(−1.7)	(−0.11)[b]
	Ethanol formed	8.3	94.4[c]

* Calculated on the basis of 100 g Initial Dry Mass of fermenting solids.
[a]Calculated as percentage of initially present.
[b]Calculated as g of biomass produced per g of carbohydrates consumed.
[c]Calculated as 100* (g of ethanol produced per g of carbohydrates consumed) / 0.56.
After Stewart et al. (*18*).

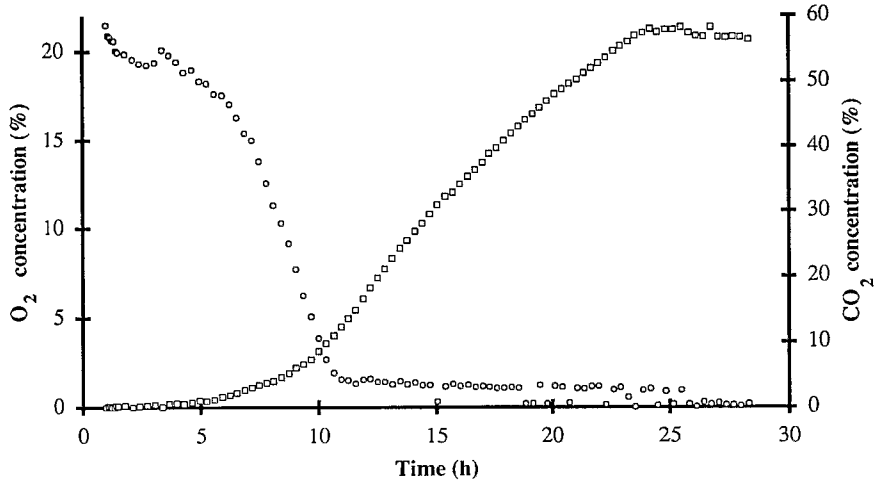

Fig. 4. Levels of O_2 and CO_2 in the exhaust air from the aerobic and anaerobic phases in a single column fermenter of 60 g moist medium capacity. ○: O_2 concentration; □: CO_2 concentration.

Ethanol Production in Anaerobic Phase

The low O_2 level and the beginning in the build-up of CO_2 concentration in the medium resulted in an anaerobic environment that shifted the metabolism from oxidative to fermentative around 10 h (Fig. 4). The data on the levels of O_2 and CO_2 in the exhaust air during the fermentation indicated the presence of a transition phase (9–11 h) between oxidative and fermentative metabolisms (Fig. 4). The production of ethanol was initiated at the end of the aerobic phase, during the transition phase itself (Fig. 3). However, no ethanol was formed before 10 h in the aerobic phase and the ethanol formation was merely 6.1 mg/g IDM at 12 h (Fig 3). The concentration of ethanol production increased progressively to the concentration of 89.3 mg/g IDM at 30 h in the subsequent anaerobic phase.

Fermentation Pattern

Data on conversion of carbohydrates into biomass and ethanol in the aerobic and anaerobic phases as well as during the whole fermentation are shown in Table 1. Results indicate that the overall ethanol conversion yields are of the order of 57.8% of the theoretical value. It is worth noticing that ethanol yield during the anaerobic phase was 94.4% of the theoretical value. Theoretical ethanol yield was calculated as 100* [g of alcohol produced per g of consumed carbohydrate] / 0.56, as indicated by Stewart et al. (*18*). The biomass formation is confined to aerobic phase for which 40% of the starch initially present in the medium is utilized. In the subsequent anaerobic phase, the biomass is just maintained and the starch

therefore is mainly consumed for ethanol production. A total of 92.9% of the starch present in the medium is utilized in aerobic and anaerobic phases of the fermentation. Similar results were also observed when the combined aerobic and anaerobic phases of fermentation were carried out in a six segmented column fermenter, except for the total absence of ethanol formation, in the aerobic phase, with the air supply rates of 0.8 and 1.5 mL/min/g wet material.

In another experiment in a six segmented column fermenter, wherein the aerobic growth of the culture was continued up to 30 h without creating anaerobic conditions, the pattern of growth and other fermentation characteristics in the first 12 h were similar to those observed above for the same fermentation period in the combined aerobic–anaerobic phases. The total carbohydrates utilized for biomass production in this case, at the end of 30 h of aerobic phase, was 98% with the biomass yield of 0.36 (g cell formed / g carbohydrates consumed). No ethanol was formed when aeration rate was 1.5 mL/min/g wet material. However ethanol was formed in the topmost segment of the fermenter between 26–30 h (10.24 mg ethanol/g initial dry matter) when the aeration rate was 0.8 mL/min/g wet material.

Characteristics of Inert Solid Support

Pith bagasse, also called as fines, is separated during depithing of the raw sugar cane bagasse (*19–21*). The depithed bagasses are used in the manufacture of paper and compressed board, whereas the pith bagasse does not find any appreciable utilization avenue, except for its use as component of animal feed in Mexico and other countries. The nonfibrous components of the pith bagasse have high specific surface and bonding power as well as low draining effects (*21*), thereby forming an excellent material for absorbing nutrient solution. It can absorb water upto 4–5 time its weight.

The pith bagasse of 0.3–0.8 mm particle size, used in the present studies as inert support, contains mainly the parenchymatous cells (Fig. 5) and a smaller proportion of broken fibers (Fig. 6). The parenchymatous cells are smaller and thin-walled as compared to the thicker walled cells of the fibers (Figs. 5 and 6). The latter have length to diameter ratio of about 70, in contrast to that of about 5 for the parenchymatous tissue of the pith (*20,21*).

Morphological Features of the Cells

The cells of *Schwanniomyces castellii* are ovoidal, globose, egg shaped or occasionally elongated as well as cylindrical and the vegetative reproduction is by multilateral budding (Figs. 7 and 8). The pseudomycelium is not formed in any of the phases of fermentation. The cell characteristics

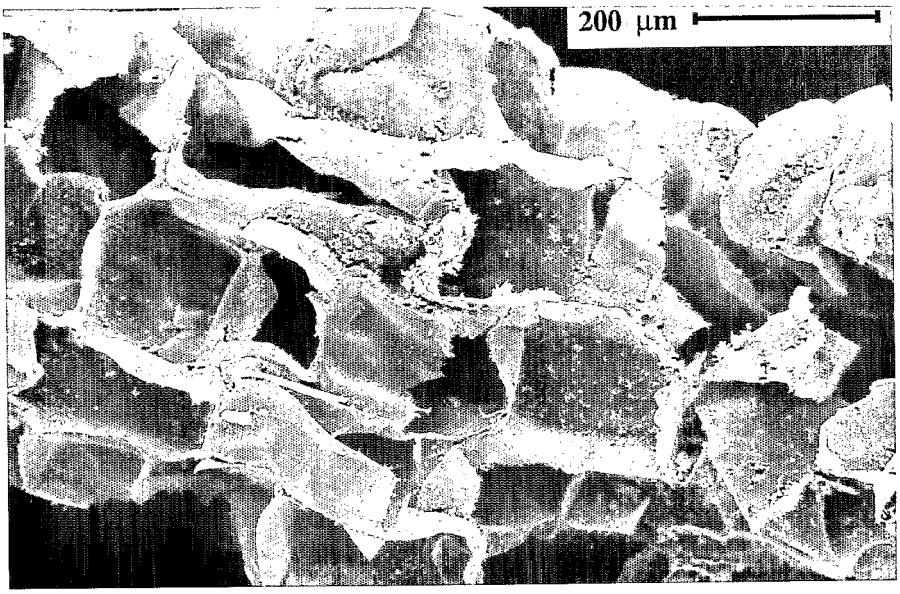

Fig. 5. Electron microphotograph of the parenchymatous cells of pith bagasse showing the development of the yeast population in the craters of bagasse cells.

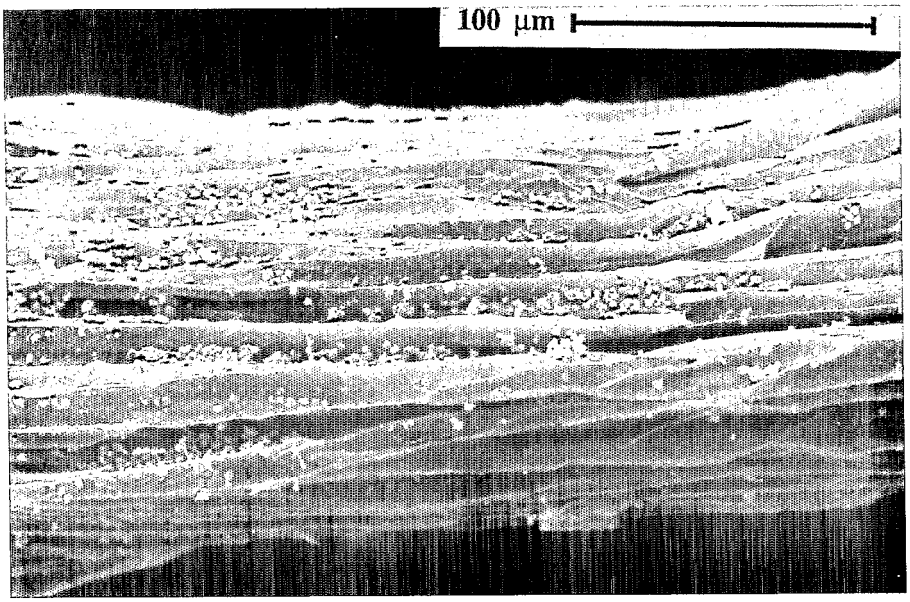

Fig. 6. Electron microphotograph of the broken fibers of pith bagasse showing the yeast culture in the hollow portions of the fibers.

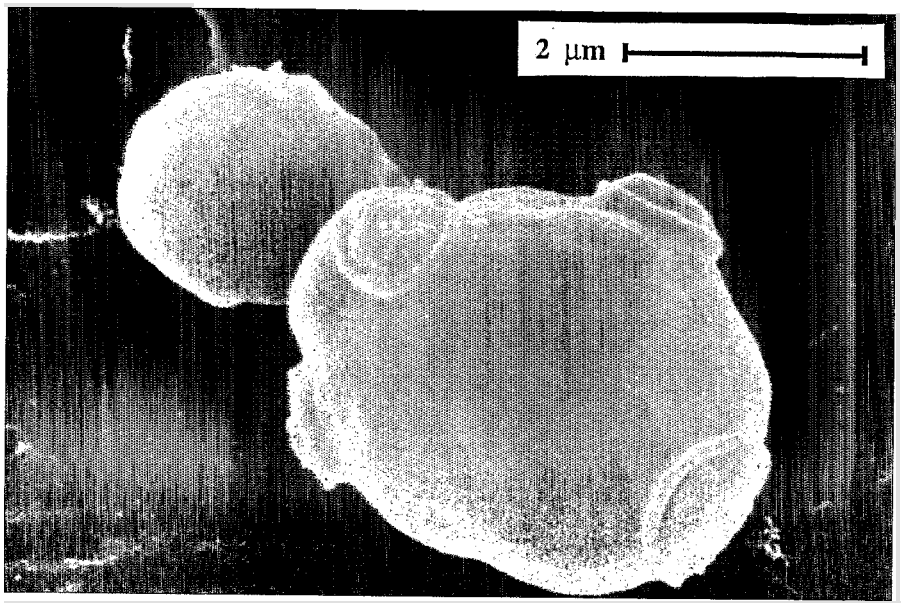

Fig. 7. Electron microphotograph of the cells from anaerobic phase of fermentation that were fixed by using glutaraldehyde.

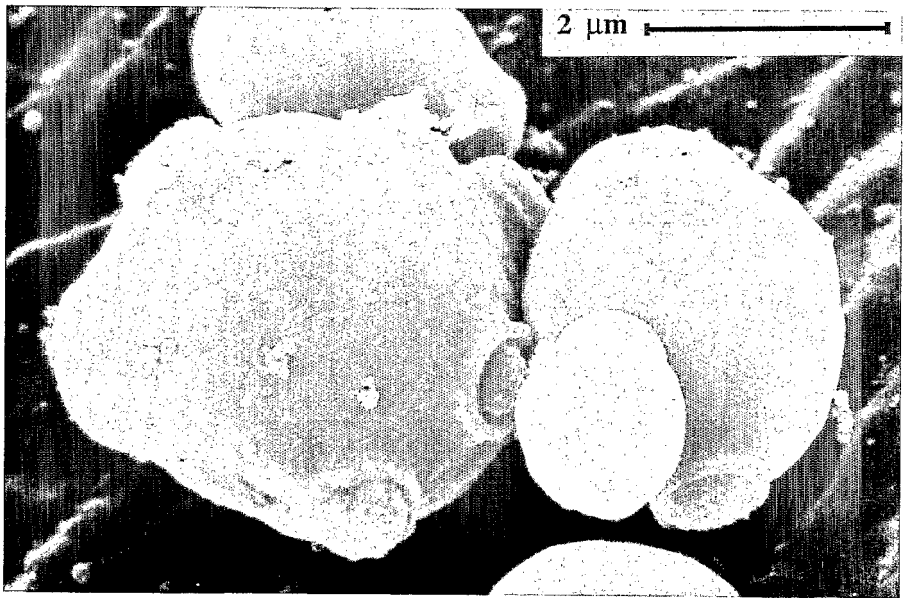

Fig. 8. Electron microphotograph of the cells from the aerobic phase of fermentation that were fixed by using formaldehyde.

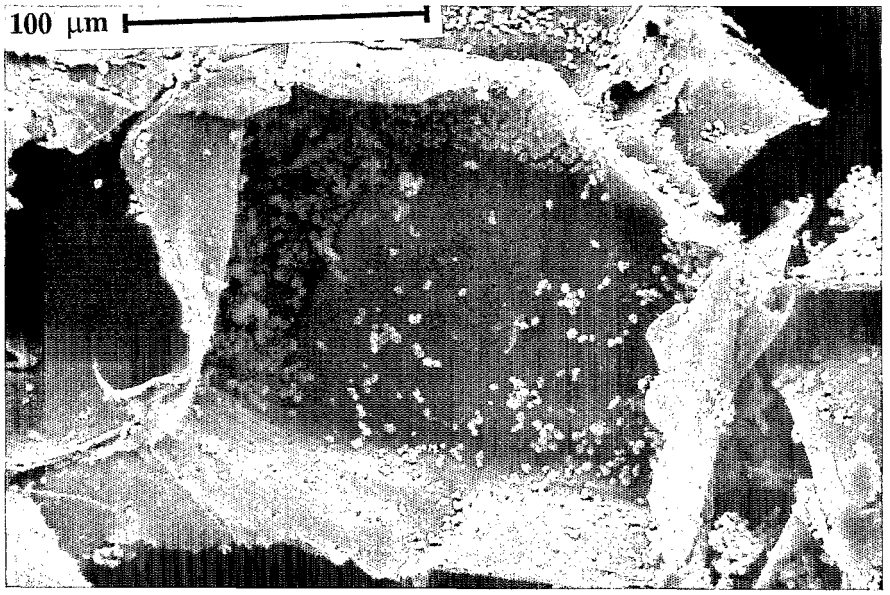

Fig. 9. Enlarged view of Fig. 5 showing the adherence of the yeast cells to the surface of the parenchymatous cells.

are exactly like those described by Phaff and Miller (22) for the genus *Schwanniomyces*. The cell shape and its morphology are similar in the aerobic and anaerobic phases of growth (Figs. 7 and 8).

It is interesting to compare the scanning electron microphotograph taken after fixing the samples using formaldehyde or glutaraldehyde. The fixation method based on the use of formaldehyde is probably too drastic, as indicated by the presence of precipitated matter on the cell surface and also the rough cell surface (Fig. 8) as compared to the cells fixed with glutaraldehyde (Fig. 7). Similar results were also noticed when the cells were from the aerobic and aerobic–anaerobic phases of fermentation (Figs. 7 and 8). The yeast cell, however, retained its turgor and natural structure in both the methods of fixation. No collapsing of the cells or disturbing of the yeast cell structure were noticed.

Cell Attachment to Solid Support

The cells were found to adhere to the surfaces of the parenchymatous tissues (Figs. 9 and 10) as well as to the surfaces of the fibers (Fig. 6) of the pith bagasses. The cell population was higher, especially in the craters created by the parenchymatous cells during the extraction of juice from the tissues in the manufacture of sugar (Figs. 5 and 9) as well as in the hollow portion of the fibers (Fig. 6). Population development seems to depend on the absorbing properties of the different types of tissues. Similar

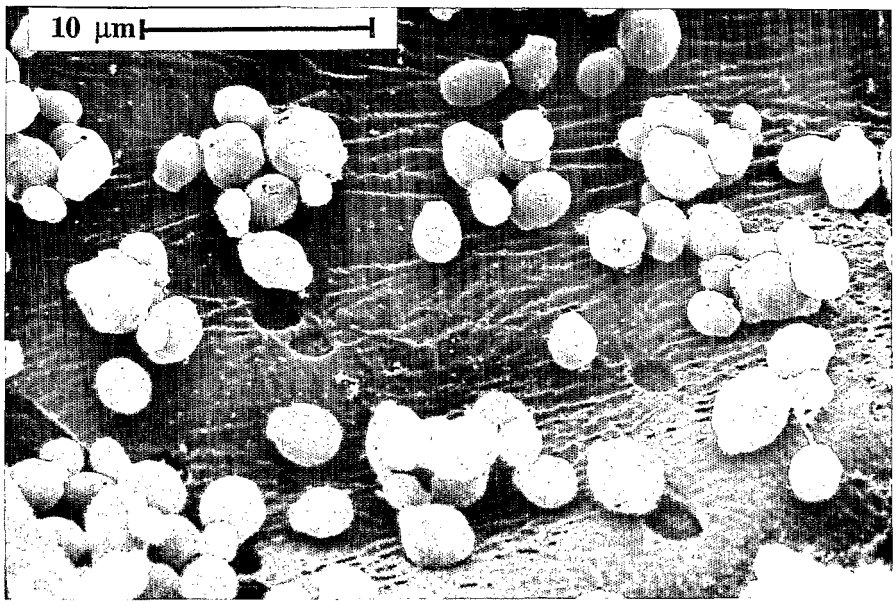

Fig. 10. The adherence of the yeast cells to the surfaces of the parenchymatous tissues.

cell adherence characteristics to the inert solid support was also observed in the cases where the culture was subjected to aerobic and anaerobic phases of the fermentation in a single fermenter.

Analysis of microphotographs indicated that the contamination chances were significantly minimized probably due to less handling of the bulky solids. Furthermore, an inoculation of 10^7 cells/mL of culture medium allows the development of *Schw. castellii* as the main microbial flora in the solid state cultures (Figs. 9 and 10).

OVERVIEWS

It is emphasized that the microorganisms in SSF systems, especially in static processes, are confined to localized regions in the moist medium (*23*), in contrast to their constantly changing location in the liquid medium owing to aeration and agitation in the submerged fermentation processes. It is for this reason that the fungi penetrate deeper into the solid particles for uptake of nutrients and the SSF system was considered to be most suited to filamentous fungi up to the beginning of the last decade (*24,25*). The lack of penetration ability in yeast, bacteria and single-celled fungi was the main reason for excluding them while studying the potentials of SSF systems (*24*). The presence of yeast and bacteria

in many food fermentations was taken as chance occurrences and novelties (*25*). Extensive efforts have been, however, put up in the recent years on growth and metabolism of yeast as well as bacteria in SSF systems (*26–30*) and resulted in good success. The growth was reported to be by adherence to the solid particles as observed by light microscopy (*31*). The present studies confirm these observations without any ambiguity. Such growth characteristics offer an easier means for biomass estimation as the cells can be easily dislodged suitably from the solids. Such biomass estimations were found to be reliable (*32*).

Saccharomyces cerevisiae with negatively charged surface was reported to adhere to support surfaces because of modification of cell and release of substances in the medium which reduce the cell-cell and cell-support electrostatic repulsions (*33*). *Schwanniomyces castellii* has been reported to form a ring over liquid medium surface (*22*), thereby indicating its ability to adhere to glass surfaces. Their adherence to the inert solid support may also be based on the above mechanisms.

The data on various aspects such as morphology of the cells, attachment of the cells to the inert solid support, patterns of biomass build-up, starch hydrolysis, glucose accumulation, and ethanol biosynthesis indicate, on the whole, the feasibility of using one single fermenter for aerobic growth of *Schwanniomyces castellii* to generate biomass (inoculum) and the subsequent anaerobic fermentation for production of ethanol.

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