KINETICS OF THE SOLID STATE FERMENTATION OF RAW CASSAVA FLOUR BY *Rhizopus formosa* 28422.

J.A. Rodriguez-Leon[1], S.C. Stertz[2], C.R. Soccol[2] and M. Raimbault[3]

[1]ICIDCA. (Instituto Cubano de Investigación de los Derivados de la Caña de Azúcar) P.O. Box 4026, La Habana, Cuba.
[2]Laboratório de Processos Biotecnológicos; UFPR / Centro Politécnico / Jardim das Américas , Caixa Postal 19011 - 081531-970 - Curitiba - PR, Brasil
[3]ORSTOM, Centre Montpellier, 911 Av. Agropolis, BP 5045, 34032 - Montpellier - France

The strain *Rhizopus formosa 28422* was selected from a stock of ten stains from genera *Rhizopus*, for their capacity to attack raw cassava starch by solid substrate fermentation and showed the highest growth in this substrate. The optimal substrate composition, estimated by surface response design experiments, was 10 % cassava bagasse, 10 % soybean flour and 80 % cassava flour. Optimal fermentation conditions were temperature, 32 °C, moisture, 64 %, initial pH, 6.5 and inoculum rate, 10^6 spores/g DM. These conditions were employed for studying the kinetics of the biotransformation of cassava flour considering the Oxygen Uptake Rate (OUR) and the CO_2 evolved during the process. The respiratory quotient was nearly 1, corresponding to an aerobic process in the first 24 h. Sporulation appeared after 26 hours of fermentation in which the respiratory quotient showed a trend to increase up to nearly 6. Biomass was estimated solving the OUR balance for a yield based on oxygen consumption ($Y_{x/o}$) of 0.531 g biomass /g consumed O_2 and a value for the maintenance coefficient (m) of the order of 0.068 g consumed O_2/g biomass^{-1} h^{-1}. The corresponding value for the growth specific velocity at the exponential phase (μ) was 0.16 h^{-1}.

Keywords : Solid Substrate Fermentation, Cassava, *Rhizopus*, Protein Enrichment, Kinetics

Introduction

Cassava flour is a basic ingredient of Brazilian people diet. However, its protein content is low (2.1% w/w, on a dry matter basis) and of poor nutritional quality (El-Dash, 1994). A biotechnological alternative for improving content and quality of cassava flour proteins is the fermentation with filamentous fungi (Vanneste, 1982). Solid State Fermentation (SSF) of cassava is a relative simple procedure, which increases by four to five folds its protein content. On another hand, the protein quality of the final product is very acceptable when compared with FAO standards (Vanneste, 1982). *Rhizopus* are edible filamentous fungi, employed since thousand of years, mainly in Oriental Countries like Chine, Korea, Japan, Indonesia, Malaysian and others, for preparing fermented foods (Hesseltine, 1965; Raimbault & Alazard, 1981). *Rhizopus* fermentation leads to protein enrichment and digestibility improvement of foods (Soccol et al. 1992, 1993, 1994). Also *Rhizopus* fermentation can restrain toxic products formation, like aflatoxins (Ko, 1974; Zhu *et al.*, 1989), produce active biocides against bacteria (Wang et al., 1969) and detoxify cyanogenic glycosides of cassava (linamarin) (Padmaja & Balagopal, 1985). As cassava has a naturally high starch content, it would be interesting to identify mould strains able to utilise this carbon source in its native forms, i. e., without the energy consuming for

gelatinization step. Thus, this research aims to develop a biotransformed flour by solid state fermentation, in order to obtain a proper protein content, employing various strains of *Rhizopus*, able to attack native cassava starch and estimate the kinetics that describe such process.

Substrate preparation : Cassava flour was prepared in the laboratory from fresh commercial roots. Roots were cleaned and handy ground. The fraction passed through 2.0 mm sieve was dried at 55-60 °C and the new fraction retained in 0.8 mm sieve was employed for fermentation studies. Cassava bagasse was purchased from Yamakawa Industries (Paranavaí, PR - Brazil). It was further ground in a disc mill (Alpha) and the fraction 0.8 - 2.0 mm was retained for assays. Soybean flour was also prepared in the laboratory, from fresh commercial beans. Seeds were toasted (10 min/250 °C), dehulled and ground. The fraction retained in 0.8 - 2.0 mm sieves was selected and employed for fermentation studies. The solid substrate was initially a mixture of 80 % cassava flour, 10 % cassava bagasse and 10% of soybean flour.

Strain: The *Rhizopus formosa* MUCL 28422 was employed due to their ability to growth in raw cassava. It was replicated in potato-dextrose agar medium, incubated during 10 days at 28-32 °C and then kept at 4 °C during six months maximum.

Inoculum preparation : Spores were first inoculated in Petri dishes containing cassava-agar medium and incubated at 28-32 °C for 10 days. Therefore, spores were collected with a platinum loop under laminar flow and diluted in test tubes with 10 mL of 1 % (v/v) Tween 80 in distillated water, previously sterilised. Spore suspension was diluted in distillated water and spores counted in a Malassez cell counter, before keeping at 4ªC.

Cassava-agar medium preparation : 30 g of cassava flour were diluted in 1 L distillated water and cooked during 1 hour in autoclave. Resulting solution was then filtered and mixed with 2.93 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.72 g urea and 15 g agar. After dissolution by heating, pH was adjusted to 5.5 with Na_2CO_3 (3N) and the final solution sterilised at 121 °C during 20 minutes.

Solid substrate fermentation conditions : The initial inoculum rate was 10^6 with an initial pH of 6.5 and an exit flow rate of 0.13 l / h g dried matter. The inlet air was saturated. The running fermentation time was 36 h at a temperature of 32 °C. The reactor was a column type with 3.5 inner diameter submerged in a water bath.

Analytical methods : Protein content was determined by the Stutzer method (Vervack 1973). Residual starch was measured by the NS-00396/85 method, employing commercial α-amylase (Thermamyl). Other parameters, like pH and moisture, ash, lipid, protein, fibber and carbohydrate contents and were determined by the Institute Adolfo Lutz Recommended Analytical Procedures (São Paulo 1985).

Results

The kinetics of the solid fermentation was determined by measuring the Oxygen Uptake Rate (OUR), the CO_2 evolved and the respiration quotient (RQ) during the process.

A balance was made for the estimation of the OUR and the CO_2 evolved in terms of volumetric flow (l/h), considering an initial weight of 27 g of dry matter, the O_2 and CO_2 percentage composition of the exhausted air flow (F_{out}) which was 0.13 l / h g initial dried weight and the inlet air flow (F_{in}) to the fermentor. The following equations were considered:

$V_{O2out} = (\%\ O_{2out} /100)\ F_{out}$

$V_{CO2out} = (\%\ CO_{2out}/100)\ F_{out}$

$V_{N2out} = (100 - \%\ O_{2out} - \%\ CO_{2out})/100)\ F_{out}$

and from the balance of O_2 and N_2 is obtained that:

$V_{O2uptake} = (20.9/100)\ F_{in} - (\%\ O_{2s} /100)\ F_{out}$

$V_{N2in} = V_{N2out}$

Relating the several equations considered, the following relationship for the inlet and outlet air flow is obtained:

$$F_{in} = \frac{(100 - \%O_2 - \%CO_2)F_{out}}{79.1}$$

For the estimation of the OUR and the C_{O2} evolved in mass flow units (mmoles/h), it was considered that the air is an ideal gas, the respective volumetric flows ($V_{O2uptake}$ and V_{CO2out}) and the proper corrections for temperature conditions, considering a temperature value of 32 °C. Table and Figure 1 show these results and the pattern of OUR and CO_2 evolved during the solid fermentation.

From Table 1 is observed that the process showed the characteristics of an aerobic system in the first 26 h with an acceptable RQ which has a mean value of 0.94 for this time interval. After 26 h this pattern holds no more and it was observed a sustained increase in the CO_2 evolved in relation to the OUR and therefor an increase in the RQ as is shown in Figure 2.

As is it observed from Table 2 there are not practically significant growth after the first 24 h with a very short lag phase of the order of only 1 h.

Table 1. OUR, CO_2 evolved and respiration quotient (RQ) pattern during the solid state fermentation of raw cassava flour by the strain *Rhizopus formosa 28422* at 32 °C.

Time	% O_2 in F_{out}	% CO_2 in F_{out}	OUR	CO_2 evolved	RQ
(h)			(mmoles/h)	(mmoles/h)	
0	20.90	0.00	0.000	0.000	
2	20.67	0.30	0.453	0.642	1.4
4	20.36	0.55	1.150	1.177	1.0
6	20.08	1.02	1.641	2.182	1.3
8	19.62	1.36	2.694	2.910	1.1
10	19.21	1.64	3.644	3.509	1.0
12	18.29	1.72	6.088	3.680	0.6
14	17.32	2.09	8.502	4.472	0.5
16	16.77	4.46	8.650	9.543	1.1
18	16.04	8.11	8.561	17.353	2.0
20	14.42	6.82	13.673	14.593	1.1
22	13.51	4.33	17.542	9.265	0.5
24	14.04	3.59	16.527	7.681	0.5
26	16.59	4.43	9.154	9.479	1.0
28	17.65	4.71	6.129	10.078	1.6
30	18.69	4.53	3.417	9.693	2.8
32	19.22	4.89	1.780	10.463	5.9
34	19.17	5.01	1.847	10.720	5.8
36	18.96	5.04	2.398	10.784	4.5

Table 2. Substrate and biomass characteristics during the solid state fermentation of raw cassava flour by *Rhizopus formosa 28422* at 32 °C.

Fermentation time (h)	0 h	24 h	36 h
Humid substrate weight (g)	75.00	70.02	69.25
Humidity (%)	64.02	64.11	66.91
initial protein due the inoculum (%)	0.12	-	-
biomass protein content (%) (d.b.)	50.16	-	-
substrate protein content (%) (d.b.)	5.58	10.13	10.68
Total biomass (g)	0.065	1.809	1.881

Based in these results it was decided to proceed with the estimation of the biotechnological parameters considering the balance of the OUR. From this balance the following equation is obtained (Sato et. al., 1983):

$$X_n = \left(Y_{x/o}\Delta t \left(\frac{1}{2}\left(\left(\frac{dO_2}{dt}\right)_{t=0} + \left(\frac{dO_2}{dt}\right)_{t=n} \right) + \sum_{i=1}^{i=n-1} \left(\frac{dO_2}{dt}\right)_{t=i} \right) + \left(1 - \frac{a}{2}\right) X_o - a \sum_{i=1}^{i=n-1} X_i \right) \Big/ \left(1 + \frac{a}{2}\right)$$

where: $a = m\,(Y_{x/o})\,\Delta t$

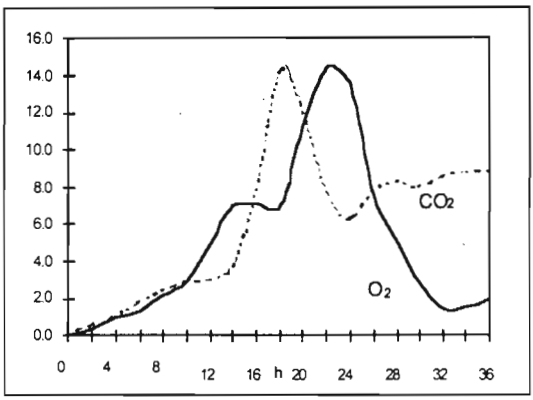

Figure 1. Kinetic pattern of the OUR and CO_2 evolved during the solid state fermentation of raw cassava flour by the strain *Rhizopus formosa* 28422.

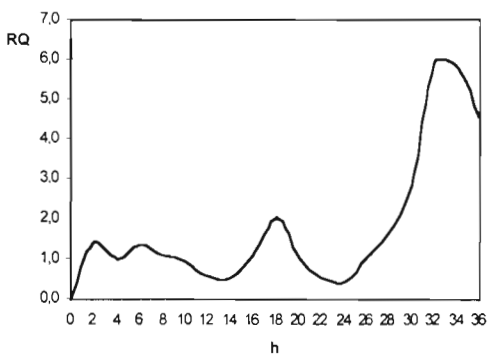

Figure 2. Respiration quotient pattern (RQ) during the solid state fermentation of raw cassava flour by the strain *Rhizopus formosa* 28422.

The procedure to estimate the biomass content in a particular moment (X_n) consist in make a trial and error estimation, assuming values for the biomass yield based in oxygen consumption ($Y_{x/o}$)

and for the maintenance coefficient (m), (Rodríguez León et al., 1988), considering in our case the biomass values analytically determined at 24 and 36 h until the values predicted by the equation here considered agree with those determined analytically. Using the data reported at Table 1 and Table 2 the system was solved for a value of 0.531 g biomass /g consumed O_2 for the biomass yield based in oxygen consumption ($Y_{x/o}$) and 0.068 g consumed O_2 / g biomass^{-1} h^{-1} for the maintenance coefficient (m).

In Table 3 are reported the biomass estimation for the different times considered, calculated via the equation for (X_n) reported before.

Table 3. Biomass estimated from the OUR during the solid state fermentation of raw cassava flour by the strain *Rhizopus formosa* 28422.

time (h)	biomass estimated (g)	biomass measured (g)	relative error (%)
0	0.065	0.065	0
2	0.067		
4	0.083		
6	0.115		
8	0.164		
10	0.237		
12	0.350		
14	0.520		
16	0.712		
18	0.891		
20	1.125		
22	1.462		
24	1.813	1.809	0.2
26	2.027		
28	2.089		
30	2.07		
32	1.996		
34	1.906		
36	1.830	1.881	2.4

Table 3 report too the comparison between the values estimates for 24 and 36 h with the values determined analytically. As it can be seen the error in the biomass estimation are lower than 2.5%. From the data of the biomass estimation it was calculated the specific growth velocity for the log phase (μ_{max}) by a regression of ln X_n vs t. The value obtained was 0.16 h^{-1} with a regression coefficient of 0.997 considering the values between 2 and 24 h.

Discussion:

The behaviour of the system after 26 h could be related to a improper air flow distribution due to air flow canalisation after the mycelium biomass was fully developed. At the same time this is a point were initial sporulation start, therefor this pattern indicate that the mycelial growth lasted until 26 h. This fact is corroborated by the results reported in Table 2 where is shown the data that characterise the substrate and biomass synthesis during the fermentation.
The value of $Y_{x/o}$ considered for solving the OUR balance seems to be relatively low and at the same time the value for the maintenance coefficient (m) seems high. This could be due to the characteristics of the substrate employed, raw cassava flour, and the necessity of synthesis by the micro-organism of the proper amylases that allow the flour hydrolysis since the beginning of the process, considering that there is not lag phase in this system, as is shown by the kinetic pattern. In this sense is notable that the pattern of enzyme synthesis seems more a constitutive and not an inductive one taking into account that the lag phase is practically null. In other words, if the amylases synthesis is inductive as it could be expected, the process in this case, with raw cassava flour and strain of *Rhizopus formosa* 28422, is really fast, provoking, a high maintenance coefficient and therefor a low biomass yield based in oxygen consumption.

The results here discussed corroborate that the process in which raw cassava flour is fermented by a solid state fermentation process with the strain *Rhizopus formosa 28422* is quite feasible and is developed in 24 h allowing the use of raw cassava flour without the necessity of previous pre-treatment as normally is done and attaching a level of protein of the order of 10% dried basis.

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