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Note

Protein Enrichment of Cassava by Solid Substrate Fermentation Using Molds Isolated from Traditional Foods

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Molds isolated from traditional foods were tested to upgrade the protein content of cassava (*Manihot esculenta*) by solid state fermentation. Initially 24 molds were screened by liquid cultivation to select those which produced high protein levels with good conversion rates. Then protein enrichment of cassava from 18 selected strains by solid substrate fermentation upgraded final protein contents of fermented products between 10 to 16.5%. Essentially, *Aspergillus* sp. strains gave the best results under the standard conditions studied.

Until recently, the alternative of producing foods by Solid Substrate Fermentation (SSF) was limited to studies related to traditional processes.[1-6] Mold-using fermentations change sensory properties, nutritive values, and preservation characteristics of foods.[7] Furthermore, the possibility of adapting artisanal processes to industrialized food,[8,9] enzymes,[10-12] mycotoxins[13,14] and feed protein[15-18] productions has also been explored.

Recently, the use of SSF for protein production from starchy substrates has been shown to be a feasible alternative for animal feeding.[16,19] Raimbault and Germon[20] devised a process to increase the protein content of cassava up to 20% in 30 h with a suitable strain of *Aspergillus niger*.[21] An important aspect is the concern for the toxicological characteristics of the fermented product. The use of a microorganism that has been used traditionally as food offers an interesting starting point. Based on our previous work,[19-22] we investigated the possibility of using strains isolated from Mexican, African, and Oriental traditional foods to upgrade the protein content of cassava by SSF. At first the strains were selected in liquid culture for their ability to convert starch into protein, then selected strains were tested for protein enrichment by SSF.

Materials and Methods

Microorganisms Thirty-two strains of molds were studied. The cultures were maintained on potato dextrose agar. Strains were isolated in our laboratory from Mexican traditional foods. Other strains isolated from traditional foods were obtained from the stock cultures of the ORSTOM Laboratory in Paris, France; MIRCEN (ASRCT Culture Collection) of Bangkok, Thailand, and from the NRRL International Culture Collection, Peoria, Il., USA.

Liquid medium cultivation The molds were grown in liquid cultivation in triplicate on a rotary shaker at 30°C for 48 h. The medium composition was: cassava meal 20 g, $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 1 g; and tap water to 1 *l*. The medium was boiled for 10 min and filtered. The pH was adjusted to 5 with H_3PO_4. One hundred milliliters were dispensed in 250-ml Erlenmeyer flasks and sterilized for 15 min at 1 bar in an autoclave. The flasks were inoculated

from a slant.

Pretreatment of raw material The cassava (*Manihot esculenta*) meal was obtained from the INIA, Huimanguillo, Mexico. The meal was sieved and the 10–50 mesh fraction was used. To 1 kg of the dried meal, water was added up to a final water content of 30%. The moist meal was cooked in the autoclave at 1 bar for 30 min and then cooled, frozen overnight, and dried at 50°C in a convection oven, by the technique previously described.[20]

Spore production for SSF inoculation A medium was prepared as follows: cassava meal, 100 g; $(NH_4)_2SO_4$, 8 g; urea, 2 g; KH_2PO_4, 4 g; and water, 230 ml. Twenty grams of this slurry were deposited in the bottom of 250-ml Erlenmeyer flasks which were capped with cotton and sterilized. The flasks were inoculated from a slant and incubated for one week at room temperature. After incubation, 150 ml of sterile water containing 0.5 ml of Tween 80 was added to the flask and stirred with a magnetic stirrer for 15 min. The spore suspension was filtered to eliminate mycelium and undigested material. The spore concentration was counted in a Neubauer Chamber. For SSF, the inoculum size was 2×10^7 spores per gram of cooked cassava.

Solid substrate fermentation The fermentation was done under non-sterile conditions as described by Raimbault and Alazard.[22] The fermentors of 20 g capacity were incubated in a 35°C water bath. Twenty fermentors could be done simultaneously with individual aeration at 4 *l*/h. The solid substrate contained 50% moisture, the pH was adjusted to 4.5, and the composition was as follows: cooked cassava meal, 100 g; $(NH_4)_2SO_4$, 9.8 g; KH_2PO_4, 4 g; urea, 2 g; and water, 95.8 ml.

Analysis The CO_2 produced by the fermentation being proportional to the respiratory metabolic activity, the amount of carbon dioxide evolved in the SSF was measured by absorbing the exit gas in an agitated flask with water at pH 11.7. The pH was kept constant by addition of a 1 N NaOH solution with a pH controller. The rate of NaOH delivery was recorded. The rate of evolved CO_2 was used to set the final time of fermentation.

Samples from the liquid fermentation were centrifuged, the supernatant kept, and the pellet washed twice with distilled water. The pellet was dried in a convection oven at 60°C. Samples from the SSF were directly dried in a convection oven at 60°C.

For the protein and carbohydrate analysis, one gram of the dried sample was homogenized with 10 ml of distilled water in a Potter homogenizer and appropriately diluted. Protein was assayed by the Folin phenol reagent method as described by Lowry *et al.*[23] Carbohydrate analysis was done using the anthrone reagent procedure.[24]

Results and Discussion

Liquid cultivation From the 32 strains which were initially selected for their ability to use starch through the iodine test, only 24 had observable growth in a rotatory shaker at 30°C after 48 h. Table 1 shows the protein production, the conversion rate of carbohydrates consumed into protein, and the origins of the tested strains.

The *Aspergillus* strains were superior in rapidly degrading starch. Good growth was also observed with the *Neurospora sitophila* isolated from *ontjom*, a *tempeh*-like fermented food that uses peanuts as substrate.[1] Strains isolated from cassava or oriental foods produced the best results. Strains 27 and 28, isolated from *pozol*, grew well in the cassava medium. In contrast, molds isolated from *pulque*[4] did not grow as well as the others.

On the basis of the protein yield and the conversion rate of starch, we selected 20 strains that used more than 70% of carbohydrate and produced more than 2 g of protein per liter (10% global protein production). For these strains, the conversion rate varied between 11.8 and 20.1 with a mean of 14.6; this rate of conversion of sugar into protein is quite acceptable for molds.

Solid state fermentation From the results obtained in the liquid fermentation experiments the 20 strains were assayed for SSF. Strains no. 25 and 28 showed no detectable growth under the tested conditions. The results are shown in Table 2. Vigorous growth was observed after the germination of the inoculated spores. The uninoculated controls showed no detectable carbon dioxide production or protein increment.

The behavior of mold growth varied sharply among the different strains. The time required for spore germination under the studied conditions varied from 10 h to 20 h. Optimal incubation time, estimated through

Table 1. Selection of food strains in liquid cultivation on cassava (20 g/l) in rotary shaker.

Strain	Origin	Protein g/l	Conversion[a] %	Yield[b] %
Aspergillus niger no. 31	UAM	3.33	93.5	17.8
Aspergillus niger no. 10	ORSTOM	3.25	95.0	17.1
Rhizopus sp. no. 28	UAM	3.02	91.0	16.6
Aspergillus awamori no. 13	MIRCEN, Bgk	2.83	70.6	20.1
Aspergillus sp. no. B1	ORSTOM	2.70	84.6	16.0
Neurospora sitophila no. 25	NRRL	2.68	95.7	14.0
Aspergillus oryzae no. M84	MIRCEN, Bgk	2.59	93.1	13.9
Aspergillus sp. no. T1	ORSTOM	2.57	87.6	14.7
Aspergillus sp. no. 14	ORSTOM	2.53	84.3	15.0
Aspergillus awamori no. 12	MIRCEN, Bgk	2.48	99.2	12.5
Aspergillus terricola no. R3	ORSTOM	2.48	81.5	15.2
Aspergillus sp. no. M101	MIRCEN, Bgk	2.41	93.1	13.0
Aspergillus sp. no. 72	ORSTOM	2.39	96.9	12.3
Monilia sitophila no. 27	UAM	2.30	70.6	16.3
Aspergillus sp. no. M147	MIRCEN, Bgk	2.25	93.4	12.0
Aspergillus niger no. 17	ORSTOM	2.24	75.4	14.9
Aspergillus usamii[c] no. M140	MIRCEN, Bgk	2.06	78.0	13.2
Aspergillus sp. no. 39	ORSTOM	2.08	82.5	12.6
Aspergillus sp. no. M82	MIRCEN, Bgk	2.04	86.4	11.8
Rhizopus sp. no. 7	UAM	2.00	74.1	13.5
A. oryzae no. 22	NRRL 1988	1.98	65.6	15.1
Penicillium sp. no. 16	UAM	1.77	43.4	20.4
Actinomucor elegans no. 24	NRRL 3104	1.63	66.8	12.2
Geotrichum condidum no. 26	UAM	1.60	36.2	22.1

[a] percentage of carbohydrate used/carbohydrate available. [b] g of protein for 100 g of consumed carbohydrate. [c] var. *shirousamii*.

the CO_2 evolution rate, varied from 25 to 48 h. But most of the strains tested showed homogeneous growth within the fermentor, and the final product had a very good appearance with a creamy color which was whiter than the color of the initial solid substrate. In the case of strains no. 7 and 27, sporulation was observed earlier in the uppermost part of the fermentor which is in contact with the air. *Aspergillus* sp. strains isolated from cassava or from oriental foods (*koji*, *tempeh*) were very efficient.

Typically, most of the 18 strains could grow well on cooked cassava; the enriched cassava composition varied from 10.9 to 16.5% in protein and from 28.2 to 45.2% in residual sugars. Eleven of these strains increased the protein content above 14%.

This result indicated that the enrichment process through SSF can be applied successfully to many mold strains, including strains from traditional foods. To choose the best strains for animal feed purposes, we need more tests like amino acid profiles, digestibility, and nutritional values. This is now in progress to select 3 or 4 strains for improvement.

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Table 2. Composition of final products after solid substrate fermentation of cassava.

Inoculum		Time (h)	Composition (% dry basis)	
Strain	Source		Protein	Total sugar
Aspergillus niger no. 10	Cassava	25	16.5	35.6
Aspergillus awamori no. 12	*Koji*	30	16.3	35.1
Aspergillus usamii no. M140	*Koji*	30	15.6	29.5
Monilia sitophila no. 27	Pozol	42	15.1	32.3
Rhizopus sp. no. 7	Cassava	48	14.9	39.3
Aspergillus oryzae no. M84	*Koji*	30	14.8	30.0
Aspergillus sp. no. B1	Banana	30	14.7	39.1
Aspergillus sp. no. T1	*Tempeh*	30	14.3	34.0
Aspergillus niger no. 31	Cassava	30	14.3	34.5
Aspergillus sp. no. 14	Cassava	30	14.2	37.9
Aspergillus terricola no. R3	*Ragi*	30	14.1	40.9
Aspergillus sp. no. M101	*Tempeh*	30	14.0	31.4
Aspergillus sp. no. 72	Banana	30	13.8	28.2
Aspergillus awamori no. 13	*Koji*	48	13.0	38.8
Aspergillus sp. no. M147	*Koji*	30	12.7	32.4
Aspergillus niger no. 17	Cassava	30	12.0	45.2
Aspergillus sp. no. 39	Banana	30	11.1	40.0
Aspergillus sp. no. M82	*Tempeh*	30	10.9	38.0
Raw cassava	—	—	2.50	90.00

Initial water content 50%, temperature of incubation 35°C.

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