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Full Length Research Paper

Protein enrichment of solid waste from cocoyam (*Xanthosoma sagittifolium* (L.) Schott) cormel processing using *Aspergillus oryzae* obtained from cormel flour

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Aspergillus oryzae **obtained from spoilt cormel flour was subjected to mutation treatments using X-rays, solar radiation and bleach. Following selection and screening of viable colonies on a medium containing *Xanthosoma* cormel solid process waste as the only carbon source, *A. oryzae* A7 which significantly (p 0.05) produced more biomass at a higher growth rate than the wild parent, was chosen for protein enrichment. Protein content of substrate enriched with the mutant fungal strain was higher than that enriched with the wild strain. Addition of $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, and urea to *Xanthosoma* solid process waste increased the growth rate of mutant, with the highest increase observed with urea. Medium amended with urea also had the highest protein level of 26.23% strain compared to a protein yield of 17.41% obtained in the control with no added nitrogen. The optimal temperature for protein enrichment was found to be 35^0C.**

Key words: *Aspergillus oryzae*, cormel, protein enrichment, waste, *Xanthosoma.*

INTRODUCTION

Xanthosoma sagittifolium (L.) Schott, commonly called cocoyam or tannia, is a tropical root crop widely distributed in West Africa, tropical America and Asia (Duru and Uma, 2002) . Cormels of cocoyam are rich sources of domestic and industrial starch. Large quantities of carbohydrate-rich solid waste are produced in the course of processing cormels into starch and this constitutes a waste disposal problem (Duru, 2000).

The changing economic, social, political and cultural values of the world have challenged all countries to efficiently use their resources. In order to comply with various environmental laws in the various countries, industries that face a problem of waste management and disposal are altering their production methods to more fully consider by-products recovery and use (Joglekar et al., 1983). Protein production by solid substrate fermentation using renewable resources including organic waste has received worldwide attention (Oliveira, 2001) as a solution to environmental and industrial waste problem. Similarly, the increasing demand for proteinaceous food and the rise in prices of products such as soy meal and fish protein over the last 10 to 15 years (Joglekar et al., 1983) have shown that there is a market for a proteinaceous food substitute. In addition, use of protein rich products as animal feed would release widely used feed materials such as soy meal, fish protein, cotton seed flour, protein isolates and peanut meal for human consumption in areas where conventional human food stuffs are in short supply (Joglekar et al., 1983). So far there is no published report on the economic use of *Xanthosoma* cormel solid process waste. This work aims at using simple solid substrate fermentation procedures

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to produce protein enriched products from solid cormel waste using *Aspergillus oryzae* (Ahlburg) Cohn. Several species of *Aspergillus* including *A. oryzae* have been previously associated with traditional fermented foods (Raimbault, 1998).

MATERIALS AND METHODS

Isolation and Media

A. oryzae were isolated from stored *X. sagittifolium* cormel flour using a single spore isolation technique in order to ensure genetic similarity. The sole carbon source used for media preparation was *Xanthosoma* solid process waste obtained from 15 kg of peeled cormels of *X. sagittifolium.* The peeled cormels were washed, cut into small pieces, blended for 3 min and the resulting slurry sieved with a muslin cloth. The filtrate was a starch-rich suspension that yielded domestic or industrial starch. The solid residue, which normally would have been discarded as waste, was the carbon source for protein enrichment. Four media formulations were used:

Media formulation A: This consisted of *Xanthosoma* solid cormel waste as carbon source with the following added:$(NH_4)_2SO_4$, 6.0 g; K_2HPO_4, 7.0 g;KH_2PO_4, 3.0 g; $MgSO_4.7H_20$, 0.1 g; KCI, 1.0 g; $FeSO_4$.$7H_20$, 0.1 g; sodium citrate, 0.5 g; and water, 200 ml. Media formulation B: This consisted of *Xanthosoma* solid cormel waste as the only carbon source, with the following added: NH_4NO_3, 6.0 g; KH_2PO_4, 1.0 g; and water, 200 ml.
Media formulation C: This consisted of *Xanthosoma* solid waste as the only carbon source plus NH_4NO_3, 6.0 g, as a nitrogen source; and 200 ml of water.

In formulations A – C, the salts were dissolved in 200 ml of water and solid waste added until the one litre mark was reached. The medium was properly mixed to ensure even distribution of the salts. Then 10 ml of the slurry mixture were dispensed into Petri dish fermenters, covered and sterilized at 121^{o}C for 15 min.

Media formulation D: In this case, 200 ml of water was placed in a beaker and *Xanthosoma* solid process waste was added to it and mixed until one litre mark was reached. Then 10 ml of this mixture were spread on the bottom of Petri dish fermenters and sterilized for 15 minutes at 121^{o}C in an autoclave.

Mutation of *A. oryzae*

A modification of the methods of Duru and Uma (2003) was used for mutation, selection and screening of the fungus. 5 ml of sterile distilled water was added and the surface of 5-day old culture agitated with sterile inoculating loop to loosen the spores and form a suspension, which was collected in a tube. The spore suspension was concentrated in a centrifuge at 550 g for 15 min and number of spores estimated. The concentrated spores were re-suspended in 10 ml of sterile distilled water and placed in a Petri dish ready for irradiation. The fungal spore suspension was exposed to the following X-ray dose treatments:

Treatment A: Four Petri dishes containing spores suspensions of the fungus were exposed to an X-ray radiation of 50 kV at 20 mAs (0.2 s X 100 mA) at a focal distance of a 95 cm and a focal spot size of 2 mm.
Treatment B: Four Petri dishes containing spores suspensions of the fungus were exposed to X-ray radiation of 75 kV at 16 mAs (0.16 s x 100 mA) at a focal distance of 95 cm and focal spot size of 2 mm.
Treatment C: Four Petri dishes containing spores suspensions of the fungus were exposed to an X-ray radiation of 100 kV at 10 mAs (0.10 s X 100 mA) at a focal distance of 95 cm and focal spot size of 2 mm.
Treatment D: Colonies of the fungus obtained after exposure of stock cultures to Treatments A, B and C were re-exposed to Treatment C.

A second set of culture was exposed to the following:

Treatment E: The fungal spores were washed with 10% bleach (Parazone), exposed to sun at 34^{o}C for 6 h and then exposed to Treatment C above.
Treatment F: Colonies obtained after exposure to bleach, sun + Treatment C were re-exposed to sun + Treatment C.

The irradiated fungus was plated out on a selection medium containing *Xanthosoma* solid waste (as the only carbon source). The inoculated plates were incubated at 35^{o}C over a period of 24 to 30 h. The colonies that developed were then sub-cultured. Mutant strains were selected based on their ability to grow on a *Xanthosoma* solid waste. Colonies that showed good growth when compared with non-irradiated controls were subjected again to the mutation process. Mutants were then selected based on growth rate, stability, biomass production and substrate utilization ability. The selected mutants were then used for protein enrichment.

Substrate Enrichment

A modification of the method of Moo-Young et al. (1992) was used for protein enrichment process. Inoculum consisted of about 1 x 10^6 spores per plate in the form of spore suspension. The fermentation process was carried out in Petri dish fermenters with the sterilized solidified substrate medium evenly spread out at the bottom. The spores dispersed in 1ml of sterile water were inoculated on the substrate, and the dish fermenter rotated to ensure even coverage of the spore suspension. This was done for media formulations A-D. The controls were not inoculated with fungal spores. Incubation was carried out at a temperature of 35^{o}C for 48 h. The resulting mycelial growth on the substrate was then mixed with the substrate beneath and dried for 2 days in a convection oven at 60^{o}C. The dried product was then ground into powder, packaged and stored for use.

Biochemical analyses

To determine total carbohydrates, a modification of the Clegg Anthrone method (Hagiwara, 1985) was used. The Anthrone reagent was prepared by dissolving 0.1 g of Anthrone in a solution of 100 ml of H_2SO_4 (specific gravity 1.84) to which 33 ml of distilled water was added. Duplicate 1 ml samples of dilutions of ethanol extract obtained from 1 g of protein enriched product were mixed with 5 ml of Anthrone reagent, heated at 100^{o}C for 12 min and read at 625 nm wavelength. Carbohydrate was determined by comparison with a standard curve prepared with glucose. Protein was determined by the Folin Ciocalteu phenol reagent method as described by Lowry et. al. (1951) using 1 g of the dried sample which was homogenized in 10 ml of distilled water. Amino acid determination was done using the method of Yemm and Cocking (1955). Absorbance was read at 470nm and concentration of amino acids was determined from a glycine standard curve. Lipids were determined using the method described by Bradbury and Holloway (1988). Ash and fibre contents were determined by the methods described by Kirk and Sawyer (1991).

Growth Characteristics

The nitrogen sources tested were 6.0 g each of NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl and Urea. Each was dissolved in 200 ml of water and *Xanthosoma* solid process waste added to it gradually until the one litre mark is reached in a beaker. The control experiment contained 200 ml of water but no added nitrogen source. Four Petri dish fermenters, each containing 11 g of substrate, were inoculated for each nitrogen source. They were incubated at 35^{O}C for 54 h, mixed, dried, ground, and protein yield determined by the methods described earlier. To determined the effects of the nitrogen-sources on the radial growth rate of fungi on solid cormel waste substrate during enrichment process, agar discs containing the protein enriching fungal strain were inoculated on substrate prepared using each of the nitrogen sources. Four Petri dishes were inoculated for each nitrogen source. They were incubated at 35^{O}C for 48 h and radial measurements were taken at 12 h intervals.

To determine the effect of temperature on protein yield, 6.0g of NH_4NO_3 dissolved in 200 ml of water was to *Xanthosoma* solid process waste added in one litre of substrate as the only added nitrogen source (and only added nutrient). This was mixed properly and sterilized. Four Petri dish fermenters, each containing 11g of the substrate, were inoculated for each temperature at 30 ^{O}C, 35 ^{O}C, 40 ^{O}C, and 45^{O}C for 40 h. The resulting mycelial growth was mixed with the substrate, dried at 60^{O}C for 2 days and ground into powder. The dried powdered samples were then analyzed to determine their protein content. The effect of incubation temperature carried out by means of agar discs containing growing fungal mutant. Four Petri dishes were inoculated for each incubation temperature, and radial measurements taken at 12 h intervals for 48 h.

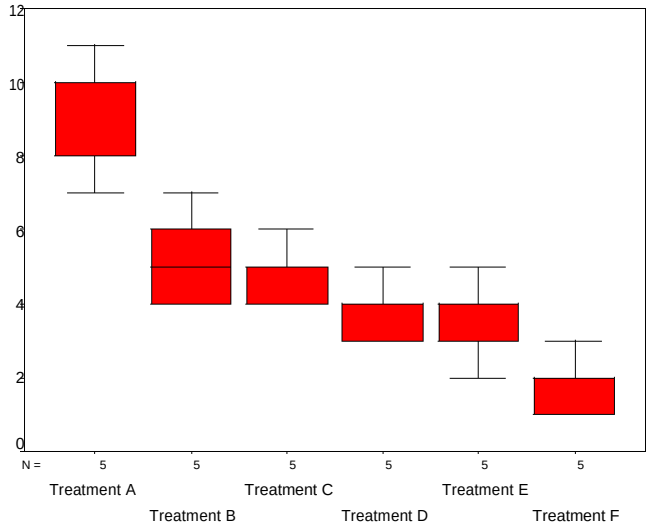

Figure 1. Mean number of viable *A. oryzae* colonies produced after each level of exposure to X-rays during strain development for protein enrichment.

RESULTS

Exposure of *A. oryzae* spores to X-radiation induced mutation in the organism. As mutation treatments became stronger, fewer colonies survived (Figure 1). The least number of colonies were produced after re-exposure to mutation process. A total of 46 colonies were produced after Treatment A while only a total of 9 colonies were produced after Treatment F. A total of seven colonies of *A. oryzae* mutants, with desirable characteristics were selected for screening for protein enrichment after mutation treatments. After screening only one mutant, *A. oryzae* A7 strain was chosen for use.

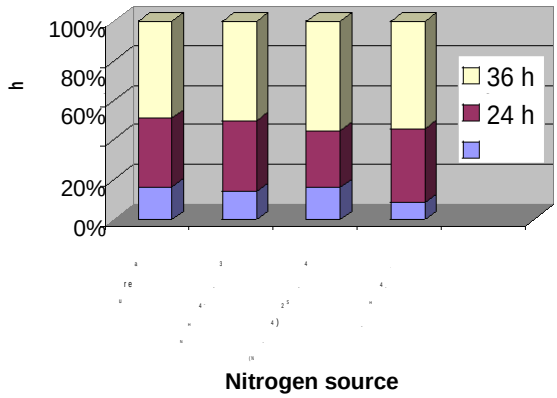

Figure 2. Effect of nitrogen source on the radial growth rate of *A. oryzae* strain A7 on *Xanthosoma* solid process waste.

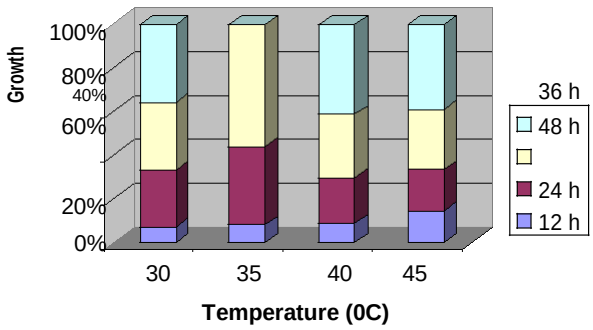

Figure 3. Effect of temperature on the radial growth rate of *A. oryzae* strain A7 on *Xanthosoma* solid process waste.

Quantity of protein produced by the chosen strain was significantly (p 0.05) higher than that of the wild strain (Table 1) in all the media tested than the wild strain. Media formulation C (containing urea) was the best medium for protein production, while media formulation A appeared to be the worst. Amino acid content, nutrient fibre and ash contents of protein enriched substrate are shown in Table 2. Addition of various nitrogen sources into solid process waste increased the growth rate of the fungus. The greatest increase in growth rate was observed when urea was added (Figure 2). In a medium amended with urea,

Table 1. Amount of protein produced on by *A. oryzae* A7 in the enriched media.

A. oryzae strain	Amount of protein in enriched substrate (g protein/g substrate)			
	A	**B**	**C**	**D**
A7	0.131 0.008	0.179 0.006	0.215 0.006	0.190 0.012
Wild type	0.107 0.004	0.151 0.008	0.142 0.005	0.134 0.010

*Media formulations were made up of: A = $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, $MgSO_4$, $7H_2O$, KCl, $FeSO_4.7H_2O$, sodium citrate and solid cormel process waste B =NH_4NO_3, KH_2PO_4 and solid cormel process waste
C =NH_4NO_3, + solid cormel process waste
D =solid cormel process waste only.

Table 2. Nutrient content of protein enriched substrate produced from *Xanthosoma* solid process waste.

Strain	**Protein**	**Amino Acid**	**Total Carbohydrate**	**Lipids**	**fibre**	**Ash**	**Residual Water**
A. oryzae A7	20.67 1.25	3.97 0.50	37.40 7.28	2.73 0.054	13.40 0.65	10.87 1.12	10.87 0.16
A. oryzae Wild	16.81 1.58	3.16 0.65	33.97 4.38	2.67 0.17	19.33 1.68	12.80 1.92	11.73 1.07
None*	1.73 0.086	0.57 0.37	16.53 1.03	1.15 0.37	48.14 3.91	16.0 1.87	15.50 1.12

*Uninoculated substrate
Amount (g) S.E

the protein level was 26.23% compared to a protein content of 1.73% obtained for the control (Table 3). Temperature affected the growth rate of *A. oryzae* A7 strain on solid waste medium. The mutant grows well at 30^oC, but grew best at 35^oC. At 45^oC it grew poorly (Figure 3). The optimal temperature was protein production was at 35^0C (Table 4).

Table 3. Effect of nitrogen source on protein content per 100g of solid substrate enriched with *A. oryzae* A7 mutant.

Nitrogen source	**Protein content (g) S.E**
Urea	26.23 0.58
NH_4NO_3	26.05 1.47
$(NH_4)_2SO_4$	22.80 0.57
NH_4Cl	22.0 0.62
Control	17.41 0.29

Table 4. Effect of incubation temperature on protein content per 100g of *Xanthosoma* solid process waste enriched with *A. oryzae* A7 mutant.

Temperature	**Protein content (g) S.E**
30^oC	17.77 0.46
35^oC	19.0 0.41
40^oC	17.92 1.91
45^oC	7.60 0.57

DISCUSSION

We have shown that a mutant of *A. oryzae* has better protein enrichment qualities than the wild strain. Exposure of stock culture to X-ray and other treatments resulted in production of fewer colonies as a result of inability of most of the spores to germinate. The fact that re-exposure of cultures further reduced the number of colonies formed suggested an increased lethality and mutation on the culture. Under natural conditions, solar radiation is the chief stimulator of photo-biological changes (Koller, 1965). Azzam (1992), working with a mixed culture of the cellulolytic fungus *Trichoderma viride* and the yeast *Candida utilis*, used acid and alkali treatment, and gamma irradiation in the enhanced production of biomass from bagasse. Hornecka et al. (1994) used multistage mutagenesis following selection to produce *A. niger* mutants over producing cellulases and characterized by poor sporulation.

Results indicate that protein enrichment of *X. sagittifolium* solid cormel process waste by *A. oryzae* A7 mutant mould strain was achieved. This study is the first report on enrichment of solid process waste of *X. sagittifolium* cormel. Cormel solid process waste often called 'chaff' in local parlance is considered worthless, as most of the starch and other nutrients have been removed leaving mainly a hardly digestible, highly fibrous material. For this solid waste material to be useful in livestock feeding it should undergo a protein enrichment process. This process in addition to enriching the substrate with protein and amino acids also releases oligosaccharides and simple sugars into the medium as a

result of microbial degradation of otherwise unavailable polysaccharides. This suggests the production of amylolytic, cellulolytic and pectinolytic enzymes in culture by the mutants that enable them to metabolize complex carbohydrate polymers. These enzymes have been reported in 'tempe' producing fungi (Graffham et al*.,* 1995). Similar studies on protein enrichment of solid waste have previously been carried out on barley, wheat and dehydrated beet pulp (Mathot and Brakel, 1991), as well as cassava (Soccol et al., 1994).

The large quantities of crude proteins produced by the fermentation of *X. sagittifolium* cormel solid process waste indicate conversion of solid carbohydrate waste into high protein fungal biomass. The crude protein obtained was comparable to similar published systems (Reade and Gregory, 1975, Jwanny et al., 1995), and the relatively high fungal growth of the mutants explains the high protein figures obtained.

Ability of the protein enriching fungus to grow at high temperatures of 35^0C and above prevents the growth of some microorganisms, which would have contaminated the process. Earlier studies on temperature indicate an optimal temperature range of 32-34.5^0C for strains of *A. niger* (Szewczyk and Myszka, 1994). The addition of exogenous nitrogen sources considerably increased biomass and protein production. Urea which is the best of the nitrogen sources tested, also helped to control pH. Roussos et al. (1994) also determined the effect of nitrogen on some fungal species and obtained comparable results. A combination of high temperature and low pH produces a highly selective growth condition, and asepsis during fermentation may be unnecessary (Reade and Gregory, 1975). The molecular characterization of the A. oryzae mutants will lead to a better understanding of the genes and pathways in the protein enrichment process.

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