*Polish Journal of Chemical Technology, 15, 2, 112 — 115, 10.2478/pjct-2013-0032*

# The use of microorganisms in increasing the protein yield of cassava (*Manihot esculenta* Crantz) peel wastes

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Growth and microbial protein production on hydrolyzed cassava peel waste by *Trichoderma viride* and *Lactobacillus delbrueckii NRRL* B-763 were investigated. *Trichoderma viride* was selected based on its high cellulase activity on filter paper (2.91 mg glucose/mL), cotton wool (3.08 mg glucose/mL) and carboxymethylcellulose (3.46 mg glucose/mL) while *Lactobacillus delbrueckii* NRRL B-763 produced 5.84 mg protein/g in cassava peel after 72 h. Samples of cassava peel were hydrolyzed with the solutions of HCl, $H_2SO_4$ and NaOH at 0.5% concentration. The hydrolysate was neutralized to pH 6.5 and supplemented with $KH_2PO_4$ (5% w/v), urea (2.7% w/v) and $(NH_4)_2SO_4$ (9% w/v). The hydrolysates produced by the solutions of HCl contained higher reducing sugar and soluble sugar content than $H_2SO_4$ and NaOH hydrolysates. The culture of *Trichoderma viride* was used in single culture fermentation of hydrolyzed cassava peels or in mixed culture fermentation with *Lactobacillus delbrueckii NRRL* B-763. Protein yield produced in 0.5% HCl hydrolysates was significantly ($p \leq 0.01$) higher than that in $H_2SO_4$. The unhydrolyzed control samples produced the lowest protein. This study demonstrated the potential of cassava peel waste as a substrate for a recycling process and by- product recovery.

**Keywords:** Cassava peel; hydrolysis methods; protein enrichment.

## INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important food crops in tropical developing countries. An estimated half-billion people in tropical and sub-tropical countries consume processed roots and leaves of cassava[1]. Cassava starchy storage roots are rich in carbohydrates but lacking in proteins. People in tropical developing countries who rely on cassava as a vital food source unfortunately suffer from protein malnutrition. In some areas of Africa, it constitutes over 50% of the daily diet of the people[2].

Numerous agricultural residues generated from diverse agricultural practices and food processing represent an important energy source. Agricultural wastes when dumped into environment may constitute a health hazard to the public. These residues can be utilized through microbial fermentation to yield food, feeds, enzymes, etc. In the processing of cassava products, the roots are normally peeled to rid them of two outer coverings: a thin brown outer covering, and a thicker leathery parenchymatous inner covering. These peels contain up to 25% cellulose[3] but are regarded as wastes and are usually discarded and allowed to rot. With hand peeling, the peels can constitute 20–35% of the total weight of the tuber[4]. The wastes generated at present pose a disposal problem and would even be more problematic in the future with increased industrial production of cassava products such as cassava flour and dried cassava fufu. One potential use of these materials is to hydrolyze them and utilize the resulting sugars. Much work has been done regarding the utilization of cassava peels as substrates for microbial protein enrichment studies[5]. The cassava peels are made up of structural macromolecules which provide an inert matrix within which the carbohydrate sources are embedded. Physical and enzymatic pretreatment help to breakdown these structural molecules, thus increasing the substrate availability to microorganisms. Pretreatment of cellulose material is useful to improve its digestibility and easy access for microbial attack. The pretreatment results in the enlargement of the inner surface area of substrate particles, accomplished by partial solubilization and/or degradation of hemicellulose and lignin. Pretreatment decreased the crystallinity of cellulose while removing lignin thereby enabling its enzymatic degradation[6]. Several physical and chemical methods are employed for cellulose pretreatment. Chemical pretreatment of lignocellulose material was developed and used extensively in paper industry for delignification of cellulose materials[7]. Chemical pretreatments improve the biodegradability of cellulose by removing lignin and/or hemicellulose and to a lesser degree, decrease the polymerization degree and crystallinity of the cellulose component[8]. The mode of action of diluted acid is to solubilize hemicellulose and lignin so that the enzymatic digestibility of cellulose is enhanced. The oligomeric hemicellulose could be completely hydrolyzed into monosaccharides by adjusting the pretreatment conditions. Alkaline pretreatment is basically a delignification process, in which a significant amount of hemicellulose is solubilized. The action mechanism is a saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and lignin. Alkaline pretreatment of cellulose materials causes swelling leading to increased internal surface area, disruption of the lignin structure and separation of structural linkages between lignin and carbohydrates[9].

The economic possibility of using cassava food rations for animal feed depends mainly on the price of cassava in relation to alterative food sources and the importance of supplementary protein sources such as animal protein[10] and amino acids[11] to be added to balance the protein requirement of cassava for animal feeding. The role of cassava as cheap carbohydrate capable of supplying adequate calories to livestock is very significant. This paper reports the increase in cassava peel protein content after their chemical hydrolysis and microbial fermentation.

## MATERIALS AND METHODS

### Materials

Cassava tubers (TMS 0581) harvested from a farm in the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria after 8–9 months of planting.

### Microorganisms used

#### Refererence lactic acid bacterial strain used:

*Lactobacillus acidophilus* NRRL B-23431, *Lactobacillus casei* NRRL B-441, *Lactobacillus fermentum* NRRL B-1932, *Lactobacillus delbrueckii* NRRL B-763, *Lactobacillus jensenii* NRRL B-4450 *and Lactobacillus brevi* NRRL B-1834 were kindly provided to the second author by Mr. James Swezey, ARS Culture and Patent Culture Collections Peoria, Illinois, USA. Inoculum was prepared from a stock culture by transferring each typed culture to an Erlenmeyer flask (250 mL each) containing 100 mL of a sterile medium described by Okafor and Ejiofor[12]: NaCl, 0.3%; $(NH_4)_2SO_4$, 0.1%; $KH_2PO_4$, 0.05%; $MgSO_4$, 0.02%; $CaCl_2$, 0.02%; lactose, 3%; linamarin (BDH, Poole England) 0.15%. The inoculum was grown at 30°C for 24 h in a Gallenkamp orbital incubator rotating at 100 x g. Cells were harvested by centrifugation using Gallenkamp Junior centrifuge at 2515 x g for 15 min. The bacterial strains were grown in hydrolyzed cassava peels and their protein yields were compared.

### Methods

#### Pretreatment of cassava tubers

Cassava peels were initially oven dried and ground to a mesh size ~ 0.5 mm. Then 100 g samples of dried, ground cassava peels were boiled for 1 h either in 400 mL of 0.5% NaOH (i), in 400 mL 0.5% $H_2SO_4$ (ii), or in 400 mL of 0.5% HCl. The alkali or acid_excess was removed by washing the treated cassava peels with sterile distilled water and the slurry pH adjusted to 6.5 with sterile lactic acid (i) or 0.2M NaOH (ii and iii). The hydrolysates were supplemented with 10 mL each of $KH_2PO_4$ (5% w/v), urea (2.7% w/v) and $(NH_4)_2SO_4$ (9% w/v). A 100 g of untreated peels were used as control.

#### Isolation of fungi

Fresh cassava tubers were cut into cubes, washed and steeped in tap water in glass bottles. The tuber cuts (10 g) were homogenized in a sterile mortar containing distilled water. The homogenate was filtered with a Whatman No 1 filter and 1 mL transferred by a sterile pipette into a 9 mL 0.1% peptone water diluents and mixed. A serial decimal dilution in 0.1% peptone water was prepared, with 0.1 mL spread onto PDA (Oxoid) and incubated aerobically at 30±2°C for 2–4 days. Pure cultures were obtained by streaking on fresh PDA plates. The cultures were assigned arbitrary numbers and stored on agar slants at 4°C.

#### Fungal selection based on cellulase activity

Czapek Dox broth (BBL Cockeysville, USA) was prepared and 5 mL added into each test tube. The tubes were sterilized by autoclaving at 121°C for 15 minutes. A loopful of each fungal isolate was inoculated into each test tube and incubated in an orbital shaker (Fisher Rotor Rack Model 343) rotating at 50 x g for 24 h at room temperature (30±2°C). This was followed by centrifugation in a Gallenkamp centrifuge at 2515 x g for 10 min. Culture supernatants (1 mL each), used for the assays, were combined with 4 mL of 0.2 M sodium acetate buffer (pH 6.5) and supplemented with either: (a) 1% (w/v) carboxymethyl cellulose (Akzo Nobel) for the carboxymethylcellulase (CMCase) activity test; (b) 50 mg Whatman No. 1 Filter paper strip (1 x 8 cm) for the filter paper activity (FPA) test or, (c) 50 mg of well-blended, high grade commercial raw cotton wool (Agary Pharmaceuticals, China) for the cotton wool activity (CWA) test and then incubated for: 60 min. at 50°C (a) and 24 h at 50°C (b and c). Reducing sugars were estimated by a modification of the dinitrosalicylic acid method of Miller[13].

#### Cultivation of microorganisms in hydrolyzed cassava peels

*Trichoderma viride* was cultured on slants of Potato Dextrose Agar. Spores were harvested with Tween 80 (Difco Laboratories, USA) solution and adjusted to $10^8$ spores/mL with sterile distilled water. Then 100 mL of the spore suspension was used to wet the cassava peels. Mixed culture media were inoculated with 50 mL each of inoculum containing 5 x $10^7$ spores/mL of *Trichoderma viride* and 5 x $10^7$ *cfu/mL of Lactobacillus delbrueckii NRRL B-763.* The experimental set up was incubated at 30±2°C for a period of 18 days at static condition.

Protein Extraction: Cassava peel (1 g) was suspended in 2 mL of 5% NaOH, 4 mL of 8% urea and 4 mL of 0.05% sodium dodecyl sulphate and incubated at 32°C for 16 h. After incubation, the samples were centrifuged and the supernatant tested for protein.

#### Assay procedures

Protein content was estimated by the method of Lowry et al.,[14] using bovine serum albumin (Sigma-Aldrich) as a standard. Reducing sugar concentrations were determined by a modification of the dinitrosalicylic acid (DNS) method of Miller[13]: DNS (10 g) was dissolved in 200 mL of 0.2 M NaOH. Potassium sodium tartrate (300 g) was dissolved in 800 mL of distilled water. The two solutions were mixed and stored in an air tight dark bottle. An aliquot (4 mL) of this reagent was added to tubes containing 1 mL of glucose solution and to distilled water blanks. The tubes were placed in boiling water bath for 10 minutes and cooled to room temperature. The solutions were read in a Spectrum Lab 23A spectrophotometer at 540 nm. The readings were used to draw a standard curve for micrograms glucose equivalents per mL against absorbance. Total soluble sugars were estimated by the Anthrone method[15].

## RESULTS AND DISCUSSION

Several fungal isolates were screened for their ability to hydrolyze cellulose substrates namely, cotton wool, filter paper and carboxyl methyl cellulose (CMC). The 14 fungal strains tested produced different cellulose activities (Table 1). Culture supernatant from the fungal isolate designated TFC 14 produced the best cellulose activities: 2.91 mg glucose/mL on filter paper; 3.08 mg

**Table 1.** Selection of fungal isolates based on their cellulase activities

| S/N | Isolate | CMCase activity [mg glucose/mL] | Cotton wool activity [mg glucose/mL] | Filter paper activity [mg glucose/mL] |
|---|---|---|---|---|
| 1 | TFC1 | 1.88 | 1.60 | 0.78 |
| 2 | TFC2 | 0.33 | 0.16 | 0.11 |
| 3 | TFC3 | 1.92 | 1.18 | 1.22 |
| 4 | TFC4 | 2.56 | 2.06 | 1.99 |
| 5 | TFC5 | 2.06 | 2.18 | 1.07 |
| 6 | TFC 6 | 1.07 | 0.69 | 0.57 |
| 7 | TFC 7 | 2.67 | 1.77 | 1.08 |
| 8 | TFC 8 | 3.12 | 2.04 | 1.74 |
| 9 | TFC 9 | 0.89 | 0.64 | 0.13 |
| 10 | TFC 10 | 0.16 | 0.11 | 0.06 |
| 11 | TFC 11 | 2.77 | 1.06 | 0.18 |
| 12 | TFC 12 | 2.90 | 0.68 | 0.40 |
| 13 | TFC 13 | 2.18 | 0.16 | 0.12 |
| 14 | TFC 14 | 3.46 | 3.08 | 2.91 |

glucose/mL on cotton wool and 3.46 mg glucose/mL on CMC (Table 1), and was therefore selected for further work. The isolate was identified as *Trichoderma viride* based on the taxonomic descriptions given by Pitt and Hocking[16] and Onions et al.,[17]. *Trichoderma* spp have been studied for cellulase production[18].

Abilities of some lactic acid bacteria to grow in hydrolyzed cassava peel waste (CPW) indicated *Lactobacillus delbrueckii* NRRL B-763 to produce the highest biomass on cassava peels and was, therefore, selected for mixed culture fermentation of the peel (Table 2). Table 2 shows the growth rates of *Lactobacillus delbrueckii* NRRL B-763 measured as an increase in protein content of the CPW after 72 h. This organism has been used for protein enrichment of cassava foods[19] and has also been used to produce lactic acid from cellulose waste materials[20, 21].

**Table 2.** Growth of Lactobacilli on cassava peel expressed as mg protein/g substrate after 72 h cultivation

| Organism | Time [h] | Crude protein [mg/g] |
|---|---|---|
| *Lactobacillus acidophilus* NRRL B-23431 | 0 | 1.81 |
| | 72 | 5.06 |
| *Lactobacillus casei* NRRL B-441 | 0 | 1.80 |
| | 72 | 3.83 |
| *Lactobacillus fermentum* NRRL B-1932 | 0 | 1.80 |
| | 72 | 3.62 |
| *Lactobacillus delbrueckii* NRRL B-763 | 0 | 1.81 |
| | 72 | 5.84 |
| *Lactobacillus brevi* NRRL B-1834 | 0 | 1.81 |
| | 72 | 4.61 |

The degradation of cellulose wastes starts with the breakdown of the polysaccharide into sugars by microbial enzymes. A variety of assays and substrates have been used for measurement of cellulose activity. Mandels and Weber[22] proposed a filter paper method for measuring the saccharifying activity of cellulase preparation. This saccharifying activity was evaluated by end point analysis of the products of saccharification using a reducing sugar assay. We evaluated the activities of cellulase by end point analysis of the products of saccharification using a reducing sugar assay. We determined a suitable hydrolysis method for improving digestibility of cassava peel waste and increasing microbial cell yield on the substrate. Acid and alkali hydrolysis were compared (Table 3). Sugar release was maximal when the peel was pretreated with HCl. Acid hydrolysis was more advantageous for producing more monosaccharides from the CPW compared to alkali hydrolysis (Table 3).

**Table 3.** Recovery of hydrolysis products of control and treated cassava peel

| Substrate | mg Carbohydrate/g substrate | |
|---|---|---|
| | Total Soluble Sugar | Total Reducing Sugar |
| Control | 62 | 38 |
| 0.5% NaOH | 210 | 142 |
| 0.5% HCl | 460 | 312 |
| 0.5% $H_2SO_4$ | 385 | 216 |

The effects of fermentation on the protein content of cassava peel are given in Figure 1. *Trichoderma viride* was used as a single culture and also used with *Lactobacillus delbrueckii* NRRL B-763 in the mixed culture fermentation. The highest protein yield from 38.2 mg/g to 175.8 mg/g substrate for single culture fermentation was produced when HCl was used for hydrolysis. The unhydrolyzed and uninoculated peel produced the lowest protein increase from 38.2 mg/g to 40 mg/g substrate (Fig. 1). For the mixed culture fermentation, the highest protein increase was from 38.2 mg/g to 203.7 mg/g produced with HCl hydrolysis of the peel. The unhydrolyzed and uninoculated peel produced the lowest protein increase from 38.2 mg/g to 40.1 mg/g substrate (Fig. 1). The protein yield produced in 0.5% HCl hydrolysates was significantly ($p \leq 0.01$) higher than that in $H_2SO_4$ and NaOH hydrolysate. This increase in the crude protein of CPW was probably due to the increase in microbial biomass as a result of microbial growth on the released sugars. The results of microbial protein yield on hydrolyzed CPW can be compared to that from other studies. Soccol et al.,[23] obtained 9.75% protein yield using *Rhizopus oligospous* grown on cassava peel. Rogers et al.,[24] reported 13.3% dry weight crude protein after 4 d growth of *Aspergillus fumigatus* on alkali-treated cellulose. Peitersen[18] obtained 21–26% dry weight (DW) crude protein by growing *Trichoderma viride* on alkali-

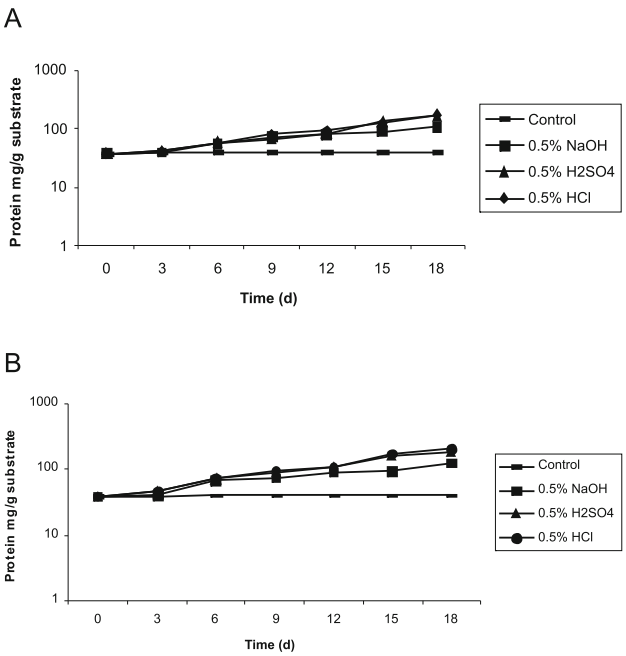


**Figure 1.** Protein yields in cassava peel wastes fermented with: A, single culture of *Trichoderma viride* and B, mixed cultures of *Trichoderma viride* and *Lactobacillus delbrueckii NRRL* B-763

-treated barley straw for 2–4 days. Romanelli et al.,[25] reported 60% utilization of fine Solka-Floc powder in 3 d by *Sporotrichum thermophile*. Eriksson and Larsson[26] obtained a product with 6% DW crude protein from powdered cellulose, 13.8% from waste fibres and 32% form highly amorphous cellulose by growing *Sporotrichum pulverulentum* for 6 d.

## CONCLUSIONS

This study showed the ability to increase the protein yield in hydrolyzed cassava peels by the growth of *Trichoderma viride* and *Lactobacillus delbrueckii* NRRL B-763. Hydrolysis method affected the concentration of sugars released and also microbial protein yield. Additional work needs to be done to determine the nutritional value of the extracted protein products as well as to evaluate the effectiveness of these processes in converting the cassava waste matter into valuable products.

## LITERATURE CITED


1. Tor- Agbidye, J., Palmer, V.S., Lasarev, M.R., Craig, M.A., Blythe, L.L. & Sabri, M.I. (1999). Bioactivation of cyanide to cyanate in sulfur amino-acid deficiency: relevance to neurological disease in humans subsisting on cassava. *Toxicological Scs.* 50, 228–235. DOI: 10. 1093/toxsci/50.2.228.

2. Oyewole, O.B. & Odunfa, S.A. (1990). Characterization and distribution of lactic acid bacteria in cassava fermentation during *fufu* preparation. *J. Appl. Microbiol.* 68, 145–152. DOI: 10. 1111/j.1365-2672.1990.tb02559.x

3. Ubalua, A.O. (2007). Cassava wastes: treatment options and value addition alternatives. *Afr J. Biotech.*6 (18), 2065–2073.

4. Ekundayo, J.A. (1980). In: J.E. Smith, D.R. Berry, and B. Kristiasen (Eds.). *Fungal Biotechnology* (pp. 244–270). London: Academic Press.

5. Aro, S.O. (2008). Imoprovement in the nutritive quality of cassava and its by-products through microbial fermentation. *African J. Biotech.* 7 (25), 4789–4797. DOI: 10.5897/AJB08.1005.

6. Aiello, C., Ferrer, A. & Bedesma, A. (1996). Effect of alkaline pretreatment at various temperatures on cellulase and biomass production with *Trichoderma reesei* QM – 9414. *Biores. Technol.* 57, 13–16. DOI: 10. 1016/0960-8524(96)00012-0.

7. Zhang, X.Y., Xu, C.Y. & Wang, X.H.J. (2007). Pretreatment of bamboo residues with *Coriolus versicolor* for enzymatic hydrolysis. *J. Biosci. Bioeng.* 104, 149–151, PMID17884661.

8. Fan, L.T., Lee, Y. & Gharpuray, M.M. (1982). The nature of lignocellulosics and their pretreatments for enzymatic hydrolysis. *Adv. Biochem. Eng. 23*: 157–187. DOI: 10. 1007/3540116982_4.

9. Rodriguez-Vazguez, R. & Diazcervantes, D. (1994). Effect of chemical solutions sprayed on sugar cane bagasse pith to produce single cell protein – physical and chemical analysis of pith. *Biores. Tech. 47*: 159–164. DOI: 10. 1016/0960-8524(94)90115-5.

10. Garcia, M. (1999). Cassava root meal for poultry. *J. Appl. Poultry Res*. 8,132–137.

11. Omole, T.A. & Sonaiya, E.B. (1981). The effect of protein source and methionine supplementation of cassava peel meal utilization by growing rabbits. *Nutr. Rep. International* 23 (4), 729–737.

12. Okafor, N. & Ejiofor, A.N. (1985). The linamarase of *Leuconostoc mesenteroides*: production, isolation and some properties. *J. Sci. Food Agric.* 36, 669–678. DOI: 10. 1002/jsfa.2740360807.

13. Miller, G.L. (1959). Use of dinitrosalicic acid reagent for determination of reducing sugar. *Anal. Chem.* 31, 426–428. DOI: 10. 1021/ac60147a030.

14. Pitt, J. & Hocking, A.D. (1997). *Fungi and food spoilage*. London: Blackie Academic and Professional.

15. Onions, A.H.S., Allsopp. D. & Eggins, H.O.W. (1981). *Smith's_Introduction to Industrial Mycology (*7th ed.) London: Edward Arnold.

16. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with folin- phenol reagent. *J. Biochem. 193*: 265–275.

17. Plummer, D.T. (1978). *An Introduction to practical biochemistry* (2nd ed.). London: McGraw- Hill Book Company.

18. Peitersen, N. (1975). Production of cellulases and protein from barley straw by *Trichoderma viride. Biotechnol. Bioeng.* 17, 361–374. DOI: 10. 1002/bit.260170306

19. Okafor, N. Umeh, C. & Ibenegbu, C. (1998). Amelioration of garri, a cassava-based fermented food by the inoculation of microorganisms secreting amylase, lysine and linamarase into cassava mash. *World J. Microb. Biobech*. 14, 835–838. DOI: 10. 1023/A: 1008897711947.

20. Shen, X. & Xia, L. (2006). Lactic acid Production from cellulosic waste by immobilized cells of *Lactobacillus delbrueckii*. *World J. Microb. Biotech. 22*: 1109–1114. DOI: 10. 1007/s11274-006-9150-4.

21. John, R.P. Nampoothiri K.M. & Pandey, A. (2007). Production of L (+) lactic acid from cassava starch hydrolysate by immobilized *Lactobacillus delbrueckii. J. Basic Microbiol.* 47, 25–30. DOI: 10.1002/jobm.200610208.

22. Mandels, M. & Weber, J. (1969). The production of cellulases. *Adv. Chem*. 95, 391–414. DOI: 10.1021/ba-1969-0095.ch023.

23. Soccol., C.R, Marin, B., Raimbault, M. & Lebeault, J. M. (1994). Breeding and growth of *Rhizopus* in raw cassava by solid state fermentation. *J. Appl. Microbiol. Biotechnol.* 41, 330–336. DOI: 10.1007/BF00221228.

24. Rogers, D.J., Coleman, E. Spino, D.F. & Purcell, T.C. (1972). Production of fungal protein from cellulose and waste cellulosics. *Environ. Sci. Terhnol. 6*: 715–719. DOI: 10. 1021/es60067a008.

25. Romanelli, R.A., Honston, C.W. & Barnett, S.M. (1975). Studies on thermophilic cellulolytic fungi. *Applied Microbiology* 30 (2): 276–281. PMCID: PMC187167.

26. Erickson, K.E. & Larson, K. (1975). Fermentation of waste mechanical figres from a news print mill by the rot fungus *Sporotrichum pulverulentum. Biotech. Bioeng.*17, 327–348. DOI: 10.1002/bit.260170304.