

FERMENTATION OF CELLULOSIC MATERIALS TO MYCOPROTEIN FOODS

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Abstract

A new bioprocess is described in which a cellulolytic, food-grade fungus *Neurospora sitophila* converts cellulosic materials to protein-rich products for food and fodder. The optimal conditions for the conversion are identified: 35-37 °C temperature, pH 5.5, 2.35 ms⁻¹ agitator tip speed. Scale-up of the production process to 1,300 L is reported. The mycoprotein production data on several types of cellulosic materials (sugarcane bagasse, corn stover, wood cellulose) are presented. The performance of *N. sitophila* is found to compare favourably with that of *Chaetomium cellulolyticum*, another cellulolytic organism previously reported on by us.

Key Words

Mycoprotein, *Neurospora sitophila*, cellulose, single cell protein, *Chaetomium cellulolyticum*.

Introduction

Cellulosic residues (e.g., straw, corn stover, sugarcane bagasse) are under-utilized by-products of the agricultural industry. Much of these residues originate from plants used traditionally in food and feed production. Because they come from acceptable food sources, the residues could potentially be upgraded to food by improvements in digestibility, nutritive value and palatability. This paper describes a new process for converting various types of cellulosic solid residues to protein-rich products for food and fodder. The process is an extension of a recent invention for converting cereal-grain bran residues into proteinaceous products (Moo-Young et al., 1990). The process is based on the filamentous fungus *Neurospora sitophila* which has a long history of use as food in oriental preparations such as *ontjom* (Hesseltine and Wang, 1967; Steinkraus, 1986; Wood and Yong, 1975). Additionally, *N. sitophila* has a processing advantage as being one of the faster growing microfungi. With a maximum specific growth rate of 0.40 h⁻¹ it has a doubling time which is shorter than that of some bacteria (Solomons, 1975). By comparison, the

maximum specific growth rates of other common industrial fungi are half (e.g., for *Aspergillus niger*) or even less than a third (e.g., for *Penicillium chrysogenum*) than that of *N. sitophila* (Solomons, 1975).

The Process Concept

Conceptually, the *N. sitophila* mycoprotein production process consists of the following steps:

- Size reduction of the cellulosic residue by milling or grinding;
- Treatment of the residue with alkali, acid and/or steam to increase the accessibility of the cellulose in the particles;
- Fermentation of the residue with *N. sitophila* either in submerged or surface culture;
- Solid-liquid separation and dehydration of the product for direct use as fodder;
- Blending, possible nucleic acid reduction, texturizing and flavouring operations for human food applications.

The size reduction, solid-liquid separation and dehydration steps use well known chemical engineering operations discussed elsewhere (Chisti and Moo-Young, 1991). The alkali pretreatment step (which, depending on the cellulosic material type, may not be needed) is described later in this article. Blending, texturizing, colouring and flavouring operations enhance the palatability and the organoleptic properties of the product. These operations are in common use in the food processing industry, and have been developed also for the fungal protein food "Quorn" being marketed in the United Kingdom (Steinkraus, 1986). Nucleic acid reduction is also used in Quorn manufacture (Steinkraus, 1986). The reduction of nucleic acids may be required if the product is used for human food. The dietary level of RNA should not exceed 2 g per day: breakdown of RNA in human body leads to elevated levels of uric acid which may cause such metabolic disorders as kidney stones. In animals uric acid is readily excreted by conversion to allantoin, and RNA reduction in feeds is not necessary. The RNA reduction techniques have been reviewed before (Solomons, 1975; Sinskey and Tannenbaum, 1975). Here we will focus only on the fermentation aspects of the *N. sitophila* mycoprotein process for upgrading celluloses.

Fermentation Process Development

Development of the process through shake flask and pilot scale fermentations was needed to answer some fundamental questions: What are the optimum pH and temperature for protein production and cellulose utilization? What are the rates and the maximal levels of protein formation and cellulose consumption? How does the utilization of cellulose - a difficult to utilize solid substrate - compare with microbial biomass production on more readily accessible

molasses? i.e., how does the "best case" protein production performance of the fungus, not limited by substrate accessibility, compare with that on less expensive cellulosic substrates? How would some of the common cellulosic residues such as corn stover and bagasse fare in terms of the ability to support protein biosynthesis? Would the fungus perform as well in fermenters as in shake flasks? What is its susceptibility to mechanical and other damage in fermenters? And, how does *Neurospora sitophila* compare with the capabilities of *Chaetomium cellulolyticum*, one of the better known cellulolytic organisms? These questions were answered by experimentation which proved the process concept and its scalability as discussed below.

Experimental

Cultures and inocula. The filamentous fungi *Neurospora sitophila* (ATCC 36935) and *Chaetomium cellulolyticum* (ATCC 32319) were maintained separately at 4 °C in submerged cultures on glucose (10 kgm⁻³) supplemented with yeast extract (Difco) (2 kgm⁻³) and the following nutrient salts (per litre): (NH₄)₂SO₄, 0.47 g; urea, 0.86 g; KH₂PO₄, 0.714 g; MgSO₄•7H₂O, 0.2 g; CaCl₂, 0.2 g; FeCl₃, 3.2 mg; ZnSO₄•7H₂O, 4.4 mg; H₃BO₃, 0.114 mg; (NH₄)₆Mo-O₂₄•4H₂O, 0.48 mg; CuSO₄•5H₂O, 0.78 mg; MnCl₂•4H₂O, 0.144 mg. Inocula were grown at 26 °C on the specified carbon source (5 kgm⁻³) supplemented with 0.5 kgm⁻³ molasses (Hoffman Feeds Ltd, Heidelberg, Ontario) and the earlier specified salts.

The fermentation media contained a carbon source ("Solka Floc" wood cellulose, sugarcane bagasse, corn stover, or molasses). The Solka Floc cellulose (α-cellulose) was made from wood pulp (James River Corporation, Berlin, New Hampshire). The KS1016 and the BW300 grades used in this work had an average particle (fibre) length of 290 μm and 22 μm, respectively. The BW300 grade had a degree of crystallinity of 62-65 % crystalline, whereas the KS1016 had a greater proportion of crystalline cellulose at 75-77 %. Apart from the Solka Floc cellulose, all other residues were pretreated with sodium hydroxide (0.15 kg/kg residue) at 121 °C for 30 minutes. Although the media were supplemented with the full complement of the earlier specified nutrient salts, only ammonium sulfate and phosphates were essential requirement with certain naturally-occurring cellulosic residues such as straw and corn stover.

Fermentation conditions. Fermentations were conducted either in shake flasks or in a 75 L (nominal) stirred tank fermenter (MBR Sulzer, Switzerland). The shake flask runs were performed in 250 mL (nominal) flasks containing 100 mL medium including an specified carbon source and the nutrient salts. The flasks were sterilized at 121 °C for 30 minutes, cooled to ambient, inoculated and held at the specified temperature on a gyratory shaker at 250 rpm. Unless otherwise indicated, the pH at inoculation was 6.0. At desired times, the flasks were

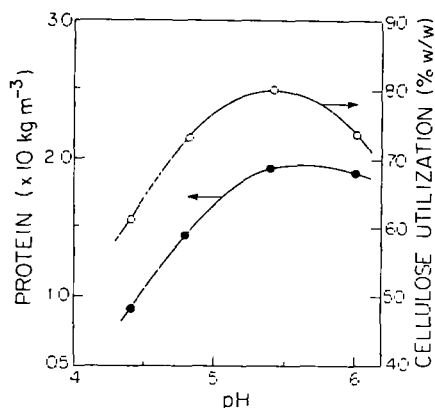
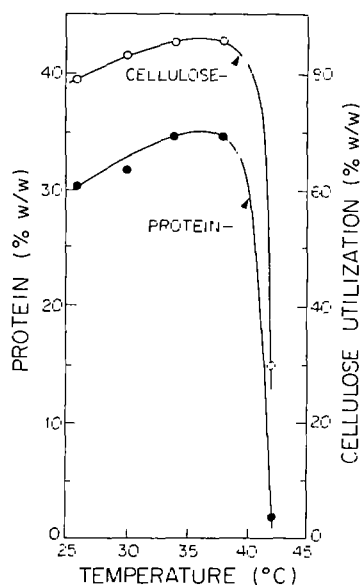
rapidly cooled and stored at 4 °C if necessary. The flasks were analyzed for total dry solids, crude protein and cellulose.

Crude protein and cellulose. For crude protein and cellulose determinations, the fermentation broth was filtered under suction through a 25 µm "Nitet" nylon cloth (Thomson Co., Scarborough, Ontario), the filter cake was washed with several broth volumes of deionized water and dried overnight at 90 °C. The dry biomass was ground to 1 mm particle size and a portion was analyzed for total nitrogen using a microKjeldahl technique (Lang, 1958). The crude protein content of the biomass were calculated as $6.25 \times$ total nitrogen, and percent (w/w) protein as gram protein per 100 g total dry solids. The cellulose content were determined by the spectrophotometric anthrone-sulfuric acid method (Updegraff, 1969); percent cellulose was calculated on the same basis as crude protein.

Shear effects. The influence of shear on protein production was investigated in the 75 L fermenter (vessel diameter = 0.318 m; working aspect ratio = 1.9) with a working volume (after inoculation) of 50 L. The temperature and pH were controlled at 26 °C and pH 6.0, respectively. The dissolved oxygen level was not allowed to drop below 20 % of air saturation. Aeration rate varied (0.4 - 0.8 vvm) in response to the dissolved oxygen level. A 6-blade disc turbine was used for agitation (diameter of impeller: tank diameter = 0.57; location above bottom of tank = 0.6 impeller diameter) at 250, 300 or 350 rpm corresponding respectively to tip speeds of 2.35, 2.82 and 3.29 ms⁻¹. *N. sitophila* was grown on Solka Floc (KS1016) (5 kgm⁻³) supplemented with molasses (0.5 kgm⁻³), (NH₄)₂SO₄ (0.28 gL⁻¹), urea (0.52 gL⁻¹), KH₂PO₄ (1.0 gL⁻¹) and other, previously listed, nutrient salts at half the concentrations specified earlier.

Results and Discussion

Temperature effects. The effect of temperature on cellulose utilization and crude protein production is shown in Figure 1. The results in Figure 1 were obtained in shake flasks with *N. sitophila* grown on industrial cellulose slurry medium (10 kgm⁻³ Solka Floc grade KS1016) supplemented with molasses (1 kgm⁻³) and the nutrient salts. Each data point in the figure (Figure 1) corresponded to the maximum cellulose utilization and protein production at the given temperature at 38 hours since inoculation (10 % v/v, ca. 0.3 kgm⁻³ initial protein) of the flasks. The fermentations peaked ca. 38 hours after initiation. A maximum cellulose utilization of ~ 86 % of original cellulose and protein production of ~ 35 % of the total dry weight were observed at 37 °C. The optimal fermentation temperature range was 35-37 °C; temperatures higher than ~ 38 °C caused sharp decline in biomass production (Moo-Young *et al.*, 1992).



◀ **Figure 1.** Effect of fermentation temperature on *N. sitophila* protein production and cellulose utilization.

▲ **Figure 2.** Effect of fermentation pH on crude protein production (*N. sitophila*) and cellulose utilization.

Effect of pH. The influence of pH on *N. sitophila* fermentations of cellulose (Solka Floc BW300) is shown in Figure 2. These fermentations were conducted in the 75 L fermenter at 37 °C. The concentration of cellulose and the supplements were the same as used in the previous set of experiments on temperature effects. The inoculum for these runs was grown on KS1016 grade of Solka Floc cellulose (10 kg m⁻³) supplemented in the same way as the fermentation medium. All fermentations were inoculated at pH 6.0, the pH was allowed to fall to one of the set points shown in Figure 2, and was controlled at the set point. The protein production and cellulose utilization reported in Figure 2 were the maximum values which occurred ~ 38 hours into the fermentation. A pH optimum of pH ~ 5.5 was identified. At this pH ~ 80 % of the cellulose which was originally present had been used up by 38 hours, and ~ 2 kg m⁻³ protein had been produced which represented ~ 33 % of dry weight of the product (Moo-Young et al., 1992).

Typically, in the ammonium/urea containing media as used in this work, the pH in fungal fermentations without pH control, initially declines due to consumption of NH₄⁺ as the nitrogen source. Upon exhaustion of NH₄⁺, urea supplies the required nitrogen and the pH of the broth rises. This behaviour is observed in *N. sitophila* fermentations (e.g., Oguntimein et al., 1992) as well as those of other fungi. The ammonium/urea ratio in the medium can be manipulated for rough pH control particularly in shake flasks; however, superior pH control, e.g., by acid/alkali addition, in large scale fermentations can enhance production rate and product yield sufficiently that such control is worthwhile even for low value products.

Agitation. Mechanical agitation in stirred fermenters is known to damage mycelial biomass and affect the yield of the product (Chisti and Moo-Young, 1989; Moo-Young and Chisti, 1988; Ujeova *et al.*, 1980). Characterization of the influence of the impeller speed on *N. sitophila* protein production was required to identify the suitable operational conditions, any scale-up limitations and the sensitivity of this particular fermentation to impeller induced shear.

The protein production profiles at various agitation rates (tip speeds) are shown in Figure 3. For otherwise identical conditions, increasing tip speed of the Rushton disc turbine impeller lowered the rate of protein production (Figure 3), and the maximum protein yield. Thus, as shown in Table 1, the maximum specific protein production rate (μ) decreased from a high of 0.09 h^{-1} at 250 rpm to a low of 0.05 h^{-1} at 350 rpm. In relative terms, the protein production rate (μ_R) at the highest rpm was only 55 % of that at the lowest agitation. Data on peak protein production and cellulose utilization (at 38 hours into the fermentation) are shown in Table 1 in absolute and relative terms. At the highest tip speed used (3.29 ms^{-1}) a distinct lag phase was noticed (Figure 3) in protein production compared to the results at lower agitation intensities.

Table 1. Effect of Impeller Speed on Protein Production and Cellulose Utilization

Impeller Speed (rpm)	Tip Speed (ms^{-1})	μ (h^{-1})	μ_R (-)	Crude Protein (%)	Cellulose Utilization (%)
250	2.35	0.09	1.0	31.1 (1) [*]	79.8 (1) [*]
300	2.82	0.07	0.78	27.7 (0.88)	69.0 (0.86)
350	3.29	0.05	0.55	21.2 (0.67)	55.6 (0.70)

* Values in parentheses are relative to the value at 250 rpm.

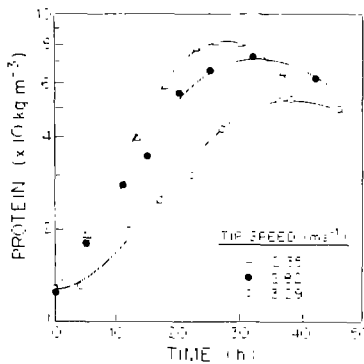


Figure 3. Effect of agitation on protein production. Impeller speed (rpm): (Δ) 250; (\bullet) 300; and (\circ) 350.

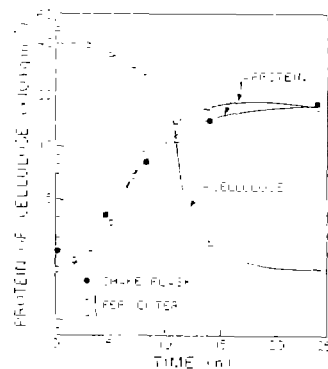


Figure 4. Comparison of protein production in shake flasks and the 75 L fermenter.

Clearly, the *N. sitophila* fermentations were quite sensitive to excessive agitation, and low agitation rates, consistent with adequate mixing and oxygen supply were indicated for the successful production process (Moo-Young et al., 1992). Further experiments (Figure 4) confirmed that an agitation speed of 250 rpm in the 75 L fermenter was optimal for protein production. At this speed the protein production in the fermenter agreed closely with that in shake flasks (Figure 4). The data in Figure 4 were obtained with *N. sitophila* grown on Chinese (Peoples Republic of China) bagasse (10 kgm⁻³) which had been pretreated with sodium hydroxide. These fermentations were conducted at 37 °C and pH 5.5.

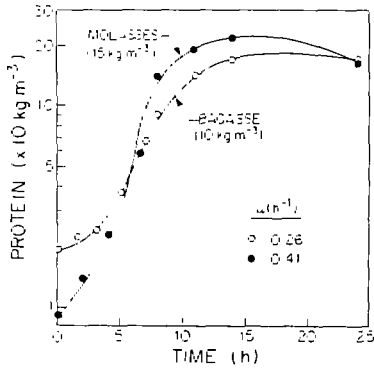


Figure 5. Comparison of *N. sitophila* protein production on molasses and bagasse in 75 L fermenter (37 °C, pH 5.5, 250 rpm impeller speed).

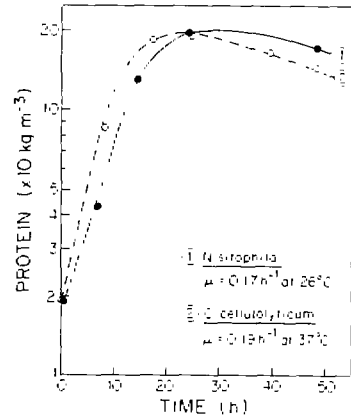


Figure 6. Protein production: *N. sitophila* vs. *C. cellulolyticum*.

Substrate characteristics. The protein production performance of *N. sitophila* on sugarcane bagasse and molasses was compared as shown in Figure 5. A specific growth rate of 0.26 h⁻¹ was obtained on bagasse, a less accessible solid substrate. On the more readily accessible molasses, the specific growth rate (= 0.41 h⁻¹) was ~ 1.6-fold greater. The growth rate on molasses was comparable to the maximum value of 0.40 h⁻¹ reported for *N. sitophila* growing on glucose at 30 °C (Anderson et al., 1975). These observations confirmed that the protein production process could potentially be improved significantly by improving the accessibility of the substrate to the fungus (Moo-Young et al., 1992).

Substrate availability was limited either by restricted physical access of the fungal cellulases to the solid particle and/or by inherent limitations in the rate of hydrolysis of cellulose. The latter could be due to either a limited rate of production of cellulases or due to limitations in the kinetics of the hydrolytic reaction itself. The possibility that secretion of *N. sitophila* cellulases and their inherent hydrolytic capability combined, were less than that of other microfungi was discounted in view of the results shown in Figure 6, where protein production

by *N. sitophila* was compared with that by *C. cellulolyticum*. The fungi were grown in shake flasks on alkali pretreated corn stover (10 kgm^{-3}) supplemented with the nutrient salts. As shown in the figure, *N. sitophila* protein production was comparable to that obtained with *C. cellulolyticum*, even though at 26°C the cultivation temperature for *Neurospora* was less than optimum. The maximum specific growth rate of *C. cellulolyticum* was about 12 % greater than that of *N. sitophila*. Both fungi had utilized ~ 93 % of the cellulose by 24 hours into fermentation (Moo-Young *et al.*, 1992). Despite these results, we believe that cellulase secretion by *N. sitophila* can be enhanced to further improve its cellulolytic potential. This view is supported by the observed two-fold increase in cellulase activity per unit biomass upon disruption of *N. sitophila* (Baldwin and Moo-Young, 1991) which implies that only about 50 % of the available cellulolytic activity is normally secreted. More detailed work on the production of cellulases by *N. sitophila*, and on the characteristics of those cellulases was reported by Oguntimein *et al.* (1992).

The product. The *N. sitophila* raw protein product had a pleasant almond smell. Almond or minced-meat flavour occurs also in *outjom* produced by fermentation of peanut press cake by *N. sitophila* (Hesseltine and Wang, 1967). The average composition of the fungus was (% w/w): 45 % crude protein, 40 % carbohydrates, 10 % fats, 5 % minerals, vitamins, etc. The amino acid composition (g per 100 g protein) of the fungal protein is shown in Table 2 where it is compared with that of fodder yeast (*Candida utilis*), soyabean meal and the FAO reference protein.

Table 2. Amino Acid Composition of Various Proteins

Amino Acid	<i>N. sitophila</i>	<i>C. utilis</i>	Soyabean Meal	FAO Reference
Threonine	3.8	5.5	4.0	2.8
Valine	6.9	6.3	5.0	4.2
Cystine	0.8	0.7	1.4	2.0
Methionine	1.8	1.2	1.4	2.2
Isoleucine	5.0	5.3	5.4	4.2
Leucine	8.0	7.0	7.7	4.8
Tyrosine	2.1	3.3	2.7	2.8
Phenylalanine	4.2	4.3	5.1	2.8
Lysine	5.3	6.7	6.5	4.2

Process Scale-up

Having identified the optimal fermentation conditions and quantified the fungal susceptibility to mechanical damage in fermenters, the fermentation process was further developed at the 75 L scale and scaled-up to 1,300 L pilot plant (Moo-Young et al., 1987). The Rushton disc turbine agitators were replaced with axial flow "Prochem" hydrofoil-type impellers (Chisti and Moo-Young, 1991) of equal diameter. A draft tube was added to enhance axial flow of the highly viscous, non-Newtonian fermentation broth (Moo-Young and Chisti, 1988) and hence the bulk mixing in the fermenter was improved. Air was sparged in the annulus between the draft tube and the walls of the fermenter. This airlift-stirred tank hybrid bioreactor proved superior to either a basic airlift or a stirred tank. Although the airlift configuration without mechanical agitation could be used (Moo-Young et al., 1987), the bulk mixing of the broth was not as effective as in the hybrid device. Unlike many viscous fermentations which have been successfully performed in airlift bioreactors (Chisti, 1989), *N. sitophila* broths containing cellulose particulates are more viscous and pseudoplastic. For example, *C. cellulolyticum* broths are rheologically easier to handle than those of *N. sitophila* (Moo-Young et al., 1987). The bioreactor scale-up considerations such as gas-liquid mass transfer and gas holdup effects in those broths have been reported on previously (Chisti and Moo-Young, 1988; Moo-Young et al., 1987). Other general aspects of fermentation plant design which apply in different degrees to food and feed plants have been detailed elsewhere (Chisti, 1992; Chisti, 1992a; Chisti and Moo-Young, 1991).

Process Economics

The economics of fungal protein manufacture were discussed in detail by Moo-Young et al. (1979; 1986) for a production processes based on *C. cellulolyticum*. Those economic analyses are equally valid for the *N. sitophila* based process because of the similarities between the two production schemes: same cellulosic substrates, media supplements, and pretreatment operations; identical downstream processing of the product and similar growth and protein production characteristics of the two fungi.

For conversion of Kraft paper pulpmill clarifier sludge (95 % cellulose) to mycoprotein, Moo-Young et al. (1986) determined that a minimum processing capacity of 6.5 tonne per day of sludge was required to break-even. A 96 % conversion of the cellulose to a product containing 38 % protein was assumed with soymeal-based protein being the reference selling price. The major contributors to production cost were the utilities (at 37.2 % of total cost), the nutrients (at 36.4 % of total cost) and the equipment depreciation (at 18.1 % of total cost).

In speciality foods industry the production cost considerations are of lesser importance than in feeds manufacture. For example, the Quorn mycoprotein is commercially produced using hydrolysed starch - a more expensive substrate than cellulosic residue - for fungal cultivation (Steinkraus, 1986). Hence, the *N. sitophila* protein process is expected to be economically viable in speciality foods markets, e.g., for vegetarians.

Conclusion

The food-grade fungus *Neurospora sitophila* was cultured on various solid cellulosic substrates (wood cellulose, sugarcane bagasse, corn stover) for mycoprotein production. The optimal conditions for cellulose utilization and protein production were found to be 35-37 °C temperature, pH 5.5 and agitator tip speed not exceeding 2.35 ms⁻¹ in a 75 L stirred tank fermenter. Up to ca. 90 % utilization of cellulose could be achieved. The cellulolytic performance of *N. sitophila* compared favourably with that of *Chaetomium cellulobiticum* which is well known for its ability to degrade cellulose. The mycoprotein production process was proven to 1,300 L pilot scale. The process is an effective method for protein-enrichment of cellulosic substances for food and fodder use.

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