

FERMENTATIVE CONVERSION OF CELLULOSIC SUBSTRATES TO MICROBIAL PROTEIN BY *NEUROSPORA SITOPHILA*

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ABSTRACT

Neurospora sitophila was used to convert solid cellulosic substrates (sugarcane bagasse, corn stover, wood cellulose) to protein-rich materials for food and fodder. The optimal conversion occurred at 35-37 °C and pH 5.5. The fungus was sensitive to excessive agitation; protein production and cellulose utilization were lowered by agitation above 250 rpm in a 75 L fermenter. The cellulolytic capability of *N. sitophila* was comparable to that of *Chaetomium cellulolyticum*, a better known cellulolytic organism.

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 the fermentative conversion of cellulosic solid residues to protein-rich materials for food and fodder. The conversion is an extension of the recently developed cereal-bran-to-protein process (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.

Development of the fermentation process through shake flask and pilot scale fermentations was done to answer questions regarding the optimum pH and temperature for protein production and cellulose utilization. The rates and the maximal levels of protein formation and cellulose consumption were determined,

and utilization of cellulose was compared with biomass production on the more readily utilized molasses. The susceptibility of the fungus to mechanical and other damage in fermenters was investigated. Finally, the cellulolytic performance of *N. sitophila* was compared with that of *Chaetomium cellulolyticum*, another cellulose-degrading organism previously studied by us (Chisti and Moo-Young, 1988; Moo-Young, 1977).

MATERIALS AND METHODS

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)₆Mo₇O₂₄·4H₂O, 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 (Huffman 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 average particle (fibre) length of 290 μ m and 22 μ m, 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 earlier specified nutrient salts, the entire complement of salts was not necessary for the naturally occurring substrates (e.g., corn stover; straw); only ammonium sulfate and phosphates were essential.

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 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.

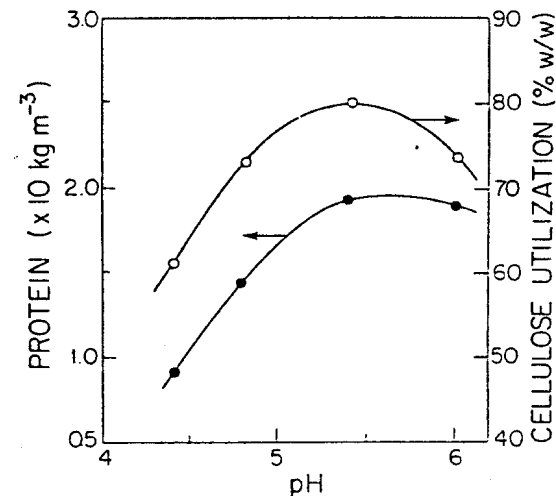
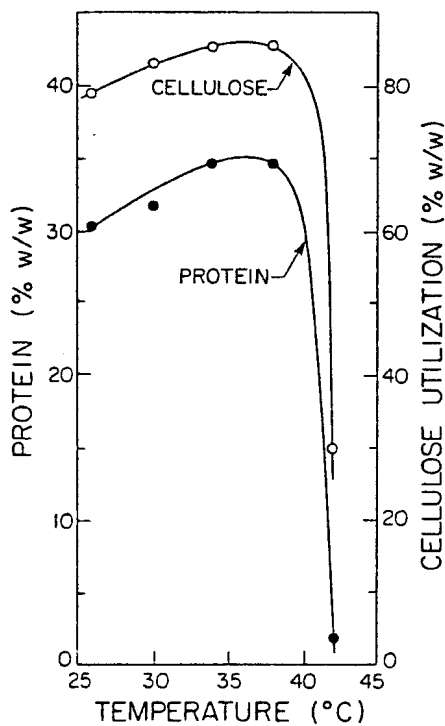
Crude protein and cellulose. For crude protein and cellulose determinations, the fermentation broth was filtered under suction through a 25 μ m 'Nitex' nylon cloth (Thomson Co., Scarborough, Ontario), the filter cake was washed with several broth volumes of deionized water and dried overnight at 90 °C. 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) with a final working volume (after inoculation) of 50 L fermentation broth. 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; working aspect ratio of tank = 1.9) 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 KS1016 grade Solka Floc (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 fermentation temperature on cellulose utilization and crude protein production is shown in Figure 1. 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 of the flasks. The fermentations peaked around 38 hours after initiation. A maximum cellulose utilization of ~ 86 % of original cellulose and protein production of ~ 35 % of the total dry weight was observed at 37 °C. The optimal fermentation temperature range was found to be 35-37 °C; temperatures higher than ~ 38 °C caused sharp decline in fungal biomass production.

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 kgm⁻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



◀ **Figure 1.** Effect of fermentation temperature on *N. sitophila* protein production and cellulose utilization. Medium: Solka Floc KS1016 (10 kgm⁻³) supplemented with molasses (1 kgm⁻³) and salts.

▲ **Figure 2.** Effect of fermentation pH on crude protein production (*N. sitophila*) and cellulose utilization. Data at 37 °C.

used up by 38 hours, and $\sim 2 \text{ kgm}^{-3}$ protein had been produced which represented $\sim 33 \%$ of dry weight of the product.

Agitation conditions. 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; Ujcova 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 Agitation on Protein Production and Cellulose Utilization

Impeller Speed (rpm)	$\mu \text{ (h}^{-1}\text{)}$	$\mu_R \text{ (-)}$	Crude Protein (%)	Cellulose Utilization (%)
250	0.09	1.0	31.1 (1)*	79.8 (1)*
300	0.07	0.78	27.7 (0.88)	69.0 (0.86)
350	0.05	0.55	21.2 (0.67)	55.6 (0.70)

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

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.

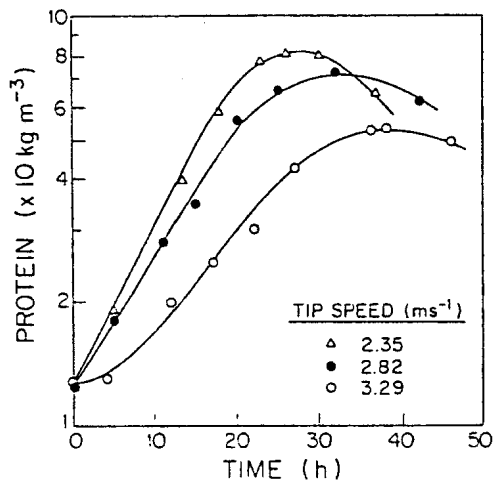


Figure 3. Effect of agitation on protein production. Impeller speed (rpm): (Δ) 250; (\bullet) 300; and (\circ) 350. *N. sitophila* grown on KS1016 grade of Solka Floc wood cellulose ($26 \text{ }^\circ\text{C}$, pH 6.0).

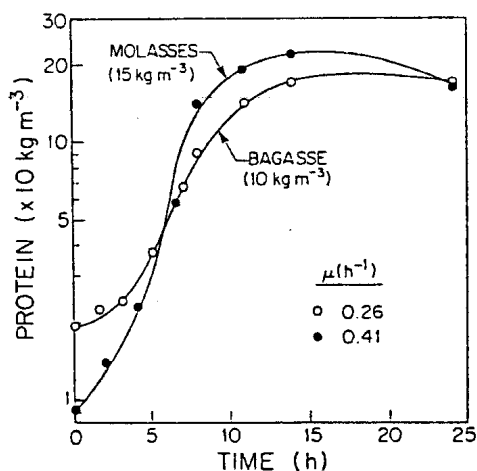


Figure 4. Comparison of *N. sitophila* protein production on molasses and bagasse in 75 L fermenter (37 °C, pH 5.5, 250 rpm impeller speed).

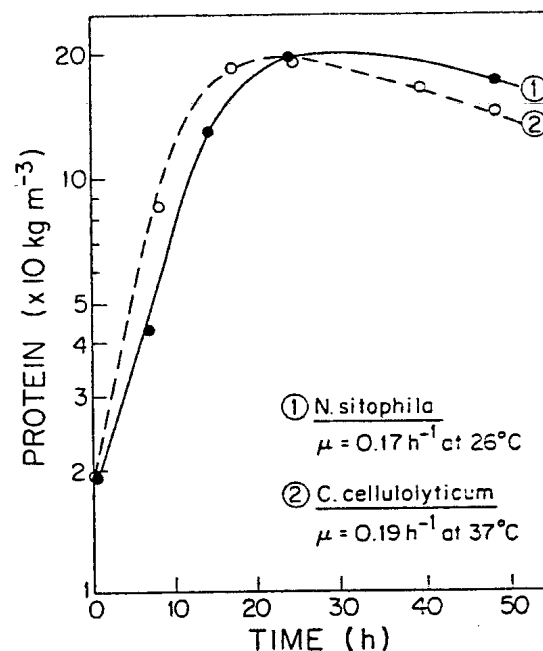


Figure 5. Protein production: *N. sitophila* vs. *C. cellulolyticum*. The fungi were grown in shake flasks on alkali pretreated corn stover (10 kgm⁻³) supplemented with nutrient salts.

Substrate characteristics. The protein production performance of *N. sitophila* on sugarcane bagasse and molasses was compared as shown in Figure 4. 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.

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 later 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 those of other cellulolytic microfungi was discounted in view of the results shown in Figure 5, 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. 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 *ontjom* produced by fermentation of peanut press cake by *N. sitophila* (Hesseltine and Wang, 1967). The mycoprotein product has potential economical applications in feed and food preparations (Moo-Young et al., 1990).

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