



# Effects of substrate particle size and alkaline pretreatment on protein enrichment by *Neurospora sitophila*

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## Abstract

The effects of solid-substrate characteristics (particle size, pretreatment conditions) on microbial biomass protein production and cellulose utilization by *Neurospora sitophila* (ATCC 36935) were investigated. Corn stover ground to various particle size fractions (<1 mm, 1–2 mm, 2–3 mm) was the test substrate. The pretreatment utilized sodium hydroxide (0–0.15 kg/kg substrate, at 121°C for 30 min) for delignification and hemicellulose removal. Cellulose utilization by the fungus and the crude protein production increased with decreasing substrate particle size and with increasing sodium hydroxide concentration in the pretreatment step. Under the best conditions, using <1 mm substrate particles treated with 0.15 kg NaOH/kg substrate, approx. 90% of the initial cellulose was consumed by the fungus and the crude protein concentration in the dry product exceeded 50% by weight.

*Keywords:* Single cell protein; Mycoprotein; *Neurospora sitophila*; Cellulose degradation

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## 1. Introduction

Protein enrichment of agricultural lignocellulosic residues (e.g., straw, corn stover, sugarcane bagasse) for food or animal feed is potentially useful in reducing the environmental impact of these residues and in enhancing animal and human food supplies. At present under-utilized, these residues can be up-graded to food by improvements in digestibility, nutritive value and palatability by fermentation with cellulolytic microorganisms [1]. Although many cellulose-degrading microorganisms, mostly fungi, are known, few would qualify as food- or feed-grade. Thus, *Penicillium funiculosum* [2], *Alternaria alternata* [3], *Trichoderma viride* [4] and *Chaetomium cellulolyticum* [5,6] are all inappropriate

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for protein enrichment processes; however, the microfungus, *Neurospora sitophila*, which has a long history of use as food in oriental preparations such as *ontjom* [7–9], is particularly suited for such a process. *N. sitophila* has been determined to have a powerful cellulolytic capability [10] which is comparable to that of *Chaetomium cellulolyticum* [1,11], a better known cellulose-degrading fungus [5,6]. Additionally, *N. sitophila* has a processing advantage as being one of the faster growing microfungi. With a maximum specific growth rate of  $0.40 \text{ h}^{-1}$  it has a doubling time which is shorter than that of some bacteria [12]. 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* [12].

A microbial biomass protein production process based on *N. sitophila* has been developed and scaled-up to 1300 l [11,13]. The bioprocessing scheme utilizes a number of general unit operations [14]. Among these, the two critically important process steps are the size reduction of the cellulosic residue by milling or grinding, and pretreatment of the residue to remove the unwanted lignin and hemicellulose prior to fermentation. These two steps have so far not been investigated in detail. Here we report on the effects of the size of solid substrate particles, and of the conditions of pretreatment, on protein production by *N. sitophila*. Optimization of the milling and the pretreatment operations is essential to the economic viability of the protein production process because these steps are energy intensive and require expensive chemicals.

## 2. Materials and methods

### 2.1. Microorganism and fermentation

*Neurospora sitophila* (ATCC 36935) was maintained at  $4^{\circ}\text{C}$  on potato dextrose agar (PDA) slants. Seed cultures were prepared in 250-ml conical flasks containing 50 ml seed medium of the following composition (per l): glucose, 10.0 g; yeast extract (Diffco), 2.0 g;  $(\text{NH}_4)_2\text{SO}_4$ , 0.47 g; urea, 0.86 g;  $\text{KH}_2\text{PO}_4$ , 0.714 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g;  $\text{CaCl}_2$ , 0.2 g;  $\text{FeCl}_3$ , 3.2 mg;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 4.4 mg;  $\text{H}_3\text{BO}_3$ , 0.114 mg;  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ , 0.48 mg;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.78 mg;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.144 mg. The pH was adjusted to pH 5.5 after sterilization at  $121^{\circ}\text{C}$  for 30 min. Flasks cooled to ambient were inoculated from PDA slants and incubated on a rotary shaker ( $35^{\circ}\text{C}$ , 220 rpm, 2 days). The biomass produced was aseptically dispersed in a blender (Waring Commercial Blender 7011, Dynamics Corporation of America, New Hartford, CT) at 'low intensity' setting for one minute and a 5 ml portion of this material was used to inoculate 50 ml production medium in 250-ml shake flasks. The production flasks were held on a rotary shaker ( $35^{\circ}\text{C}$ , 200 rpm) until desired. At desired times, the flasks were removed from the shaker, rapidly cooled and stored at  $4^{\circ}\text{C}$  if necessary. The flasks were analyzed for total dry solids, crude protein and cellulose. All flasks were run in duplicate and the data were averaged. The production conditions (temperature, pH) used had earlier been established to be optimal for *N. sitophila* culture [1,11].

The production medium contained no yeast extract or glucose. Instead, ground corn stover ( $10 \text{ g l}^{-1}$ ) was the carbon source; other medium components were the same as in the seed medium described earlier. Although the media were supplemented with the full

complement of the earlier specified nutrient salts, only ammonium sulfate and phosphates were essential requirement with corn stover, a natural substrate which contains other trace nutrients [11].

## 2.2. The carbon source

Corn stover (corn stalk and leaves) collected from a farm in Waterloo, Ontario, was ground in a Wiley mill (Thomas-Wiley Laboratory Mill, Model 4, Arthur H. Thomas Company, Philadelphia, PA) to a particle size of  $\leq 3$  mm. This material was sieved through a 2 mm (12 mesh) sieve and further sieved through a 1 mm (20 mesh) sieve to give three particle size fractions: 2–3 mm, 1–2 mm and  $< 1$  mm. These fractions were either pretreated with sodium hydroxide or used without any further treatment. When pretreated, sodium hydroxide was added to corn stover at one of three concentrations of sodium hydroxide (kg NaOH/kg substrate): 0.15, 0.10 or 0.05 (the corresponding volumetric concentrations of alkali were (kg NaOH  $\cdot$  m<sup>-3</sup> solution) 10.0, 6.6 and 3.3, respectively). The mixture was autoclaved (121°C, 30 min) and cooled to ambient. The cooled slurry was washed with 15–20 volumes of deionized water until the pH of the wash became neutral. The intention was to modify the structure of the substrate by removal of some lignin and hemicellulose. The treated corn stover was dried (95°C, overnight) and crumbled prior to formulation in the media.

In one experiment a slightly different pretreatment scheme was employed: ground corn stover ( $< 1$  mm) was autoclaved with 0.10 kg NaOH/kg substrate at the conditions specified above, the pH was adjusted after cooling to room temperature, but the deionized water wash was omitted. The remaining nutrients were added (see above), the material was autoclaved and used as described under 'microorganism and fermentation.'

## 2.3. Crude protein and cellulose

For crude protein and cellulose determinations, the fermentation broth was filtered under suction through a 25  $\mu$ 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  $\leq 1$  mm and a portion was analyzed for total nitrogen using a micro-Kjeldahl technique [15]. 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 [16]; percent cellulose was calculated on the same basis as crude protein. Percent cellulose utilized was the difference between initial and final percentages of cellulose.

## 3. Results and discussion

Utilization of cellulose as a function of fermentation time is shown in Fig. 1 for the 0–1 mm corn stover particles treated in various ways. Both the rate of cellulose consumption by the fungus and the overall extent of its utilization were enhanced with increasing con-

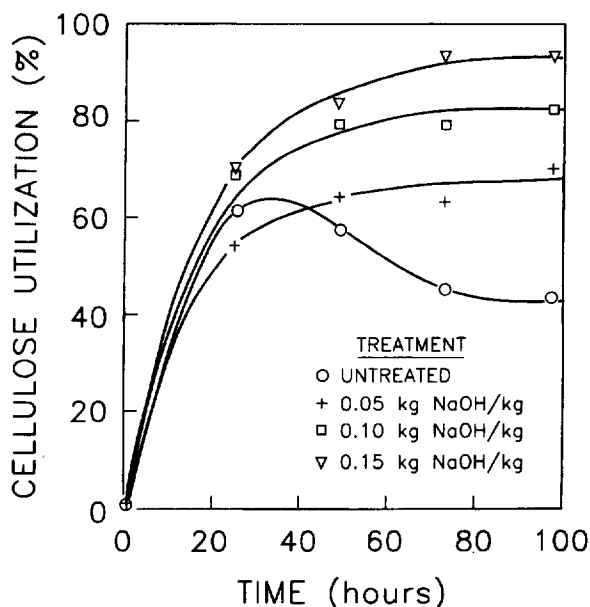


Fig. 1. Effect of different pretreatments on cellulose utilization during fermentation of < 1 mm substrate particles.

centration of alkali in the pretreatment step (Fig. 1). The maximum final cellulose utilization was 28–77% higher (depending on the pretreatment) with the treated substrate relative to the untreated base case (Fig. 1). For the best case, which used 0.15 kg NaOH/kg substrate for pretreatment, the final cellulose utilization exceeded approx. 90% of the initial cellulose. The improvements in the rate and the extent of cellulose degradation with alkali pretreatment were associated with improvements in accessibility of the cellulases to cellulose which resulted from increasing delignification and hemicellulose removal. The results (Fig. 1) implied that even in the 0–1 mm particle size range, the restricted physical access of the enzyme to the substrate was a limiting factor in degradation of cellulose in solid substrates.

With the untreated substrate, the high apparent cellulose utilization during early part of the fermentation (Fig. 1) was an artefact of the measurement method. While the total available cellulose was determined on an alkali treated sample (0.15 kg NaOH per kg substrate) of the substrate and, hence, was the same for all fermentations, low cellulose values were apparently measured with the untreated substrate possibly because the presence of other carbohydrates and lignin hid some of the cellulose from measurement in the untreated material. As the fermentation progressed and the substrate structure opened up, more of the remaining cellulose became measurable. Thus, for untreated particles, the cellulose utilization values obtained toward the end of the fermentation better reflected the actual utilization of cellulose than data obtained during the 20–60-h period.

The final yield of the crude protein increased with increasing concentration of alkali during pretreatment as shown in Fig. 2 for 0–1 mm substrate particles; however, the initial production rate was not affected very much (Fig. 2). This meant that although the cellulose solubilization rates were enhanced (Fig. 1), further breakdown of soluble polysaccharides to simple sugars was limiting the growth rate. Nevertheless, for the best case (Fig. 2), crude

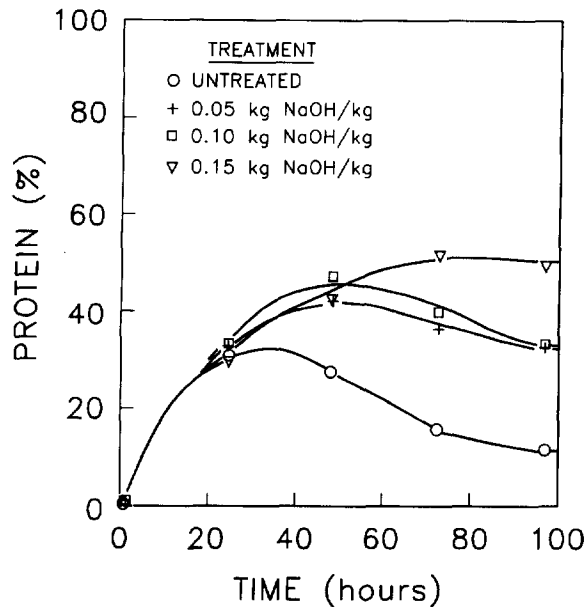


Fig. 2. Effect of sodium hydroxide pretreatment on crude protein production during fermentation of < 1 mm substrate particles.

protein made up approx. 50% (by weight) of the dry fermentation product within approx. 75 h of fermentation. Note that when soluble carbon sources such as glucose or molasses were used, the maximum specific growth rate of *N. sitophila* approached approx.  $0.40 \text{ h}^{-1}$  [1,11,12] and, starting with the same conditions as used in this work, a 50% protein buildup could be achieved in fewer than 75 h [1]. This was further evidence for limited availability of assimilable sugars as the cause of growth limitation in the solid-slurry fermentations.

The effect of size of the solid substrate particles on cellulose utilization is shown in Fig. 3. Although size reduction noticeably improved cellulose degradation in untreated (Fig. 3a) as well as alkali-pretreated (Fig. 3b) particles, this effect was small in the 0–3 mm size

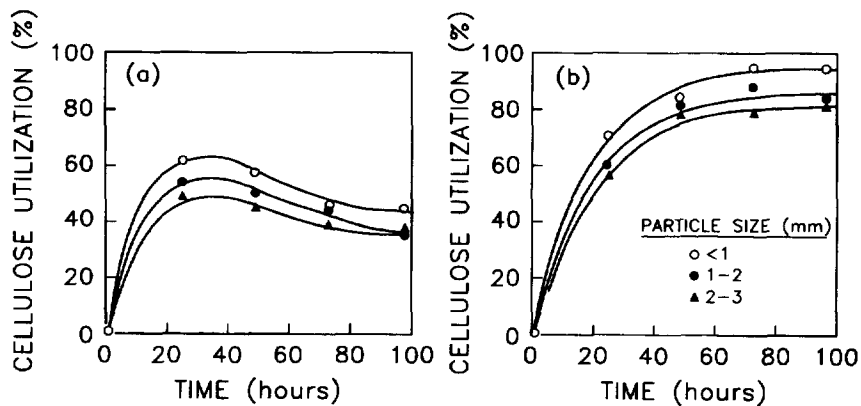


Fig. 3. Cellulose utilization as a function of fermentation time for various sizes of solid substrate particles: (a) no pretreatment; (b) pretreated with 0.15 kg NaOH/kg substrate.

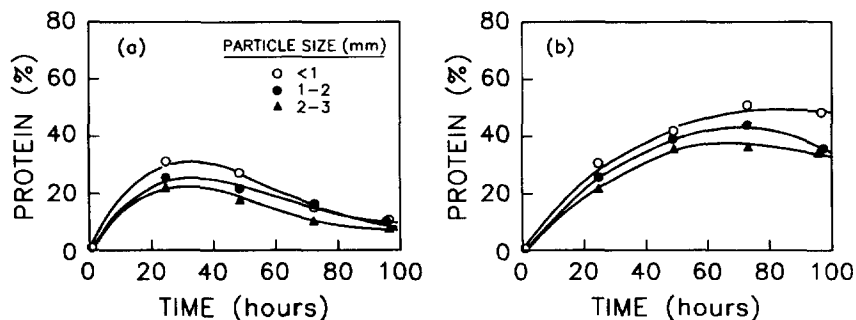


Fig. 4. Crude protein production as a function of fermentation time for various sizes of solid substrate particles: (a) no pretreatment; (b) pretreated with 0.15 kg NaOH/kg substrate.

range in comparison with the effect of alkali pretreatment (0.15 kg NaOH/kg substrate). Thus, for the milled and alkali treated particles (Fig. 3b), the maximal cellulose utilization was only approx. 16% higher for the 0–1 mm particles in comparison with the 2–3 mm particles. On the other hand, even for the larger 2–3 mm particles, alkali pretreatment with 0.15 kg NaOH/kg substrate enhanced cellulose utilization by approx. 98% relative the untreated case (Fig. 3). These results implied that in comparison with milling, the alkali-pretreatment was more effective in opening up the solid substrate particles to cellulases. For the untreated substrate, higher than actual cellulose utilization was measured during the early part of fermentation (Fig. 3a). This behaviour has been explained earlier for Fig. 1.

The crude protein as a function of fermentation time for substrate particles of various sizes, with and without alkali pretreatment, is shown in Fig. 4. The protein production, in particular the final yield, were improved by particle size reduction (Fig. 4b) as well as by alkali pretreatment. The decline in the final protein yield with fermentation time (Fig. 4a) for the untreated substrate particles arose, because by approx. 35 h of fermentation all the readily accessible cellulose had been converted to sugars, lignin and hemicellulose limited further breakdown of the cellulose and the protein yield declined due to starvation associated lysis of the biomass. Once again, although particle size reduction on the untreated substrate enhanced protein yield, the enhancement was not as much as could be achieved by treatment with 0.15 kg NaOH/kg substrate (Fig. 4). Earlier observations had indicated that the protein production could potentially be enhanced by improving the accessibility of the substrate to the fungus [11]. Substrate availability was believed to be limited either by restricted physical access of the fungal cellulases to the solid particle and/or by inherent limitations in the rate of hydrolysis or solubilization of cellulose [1,11]. In view of the results of this study, the kinetics of the hydrolytic reaction did not seem to be a limiting factor. Note that the secretion of *N. sitophila* cellulases and their inherent hydrolytic capability combined, have already been shown to be comparable to those of other such cellulolytic microfungi as *Chaetomium cellulolyticum* [1,11] and *Trichoderma reesei* [6].

Among the pretreatment options, the omission of the water wash step following alkali treatment reduced handling, simplified pretreatment, reduced water consumption and potential water pollution problems at larger scales; however, the products of degradation of lignin and hemicellulose and any soluble sugars resulting from alkaline hydrolysis of cellulose, remained in the treated substrate. As shown in Fig. 5, the breakdown components of lignin and hemicellulose had no adverse effect on cellulose consumption or protein production by

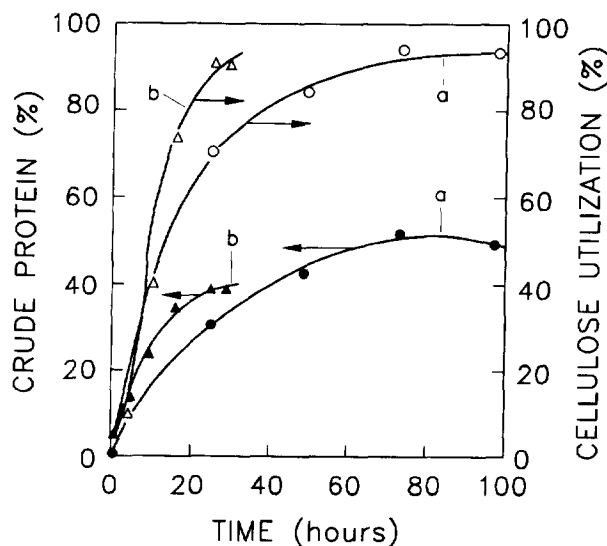


Fig. 5. Cellulose utilization and crude protein production during fermentation of < 1 mm substrate particles: (a) pretreated with 0.15 kg NaOH/kg substrate; (b) pretreated with 0.10 kg NaOH/kg substrate using a modified scheme omitting the wash step.

the fungus. In fact, the cellulose utilization and protein production were significantly enhanced in comparison with data obtained on corn stover which had been washed after the alkali pretreatment. This enhanced performance was associated with the initial availability of soluble sugars to the microorganism grown on the unwashed substrate. Note that the pretreatment omitting the water wash utilized a lower concentration of alkali (0.10 kg NaOH/kg substrate) than the 0.15 kg NaOH/kg substrate used with the washed substrate (Fig. 5). Clearly, the water wash eliminated the beneficial effects of higher alkali concentration during pretreatment. The fermentation omitting the wash step (Fig. 5) was conducted at 37°C vs. the 35°C used in all other experiments reported here. In view of the known effects of temperature on *N. sitophila* fermentations [1,11], the marginally higher temperature did not explain the dramatic improvements in cellulose degradation and protein production which were observed with the alkali-treated, unwashed, substrate.

#### 4. Conclusion

Mycoprotein production with the food-grade fungus *Neurospora sitophila* cultured on ground corn stover was studied. The effect of substrate particle size (0–3 mm) and pretreatment with various concentrations of sodium hydroxide (0–0.15 kg NaOH/kg substrate) were investigated. Both the rate and the overall conversion of cellulose to fungal protein were enhanced by reduction in size of the solid substrate particles as well as by increasing the concentration of sodium hydroxide in the pretreatment step. In the 0–3 mm particle size range, the impact of milling on improving cellulose utilization was relatively less than that of hydroxide pretreatment with 0.15 kg NaOH/kg substrate, i.e., size reduction caused less

dramatic enhancement in cellulose utilization than did alkali pretreatment. It seems, therefore, that for small particles the effect of lignin and hemicellulose in reducing the accessibility of cellulases to cellulose is more pronounced than the particle size-associated reduction in enzyme-substrate interactions. Furthermore, the results confirm that even for small (< 1 mm) cellulosic substrate particles in well agitated slurry fermentations the liquefaction of the substrate by cellulases is limited by steric hinderance or transport effects; reaction kinetics do not seem to be the limitation. Up to approx. 90% utilization of cellulose could be achieved under the best conditions (< 1 mm substrate particles pretreated with 0.15 kg NaOH/kg substrate at 121°C for 30 min; culture at 35°C, pH 5.5) when the crude protein concentration reached up to approx. 50% (by weight) dry solids.

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