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Assessing Various Types of Fungal Strains to Convert Soybean Processing Industry Wastewater into Protein-Rich Animal Feed

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Abstract

The aim of this study was to convert a soybean processing industry wastewater stream into a protein-rich animal feed via fungal bioprocessing while simultaneously reducing the potential environmental impact of the waste stream. Eight fungal strains were screened in flask trails for their ability to produce protein-rich biomass while simultaneously reducing solids found in the wastewater stream. *Trichoderma reesei*, *Paecilomyces variotii* and *Neurospora crassa* produced 51.7, 47.1 and 43.2 g/L of biomass in flask trials while reducing solids present in the supernatant fraction by 46.5, 48.9 and 49.1%, respectively. In bioflo fermenters, *Trichoderma reesei* and *Neurospora crassa* produced 55.5 and 62 g/L of protein-rich biomass while reducing chemical oxygen demand (COD) levels by 10.53 and 23.04%, respectively. Microbial metabolic process led to the production of protein-rich animal feed and simultaneously reduced the level of organic matter in the wastewater stream.

Keywords: Single-cell protein; Fungal incubation; Chemical oxygen demand; Wastewater utilization; Animal feed

Introduction

Biological wastewater streams continue to be a major economic and environmental factor in bioprocessing industry [1,2]. Conventional methods used over the past century such as aerobic and anaerobic wastewater treatments utilize bacteria to reduce organic matter present in these streams to acceptable levels [3]. These methods possess little benefit for companies as focus is on removing necessary amounts of organic material to meet legislative demands on disposable wastewater [4,5]. As a result of these processes, a large amount of low-value bacterial biomass otherwise known as "sludge" is produced which can account for up to 60% of wastewater plant operating costs when it needs to be discarded [6]. Alternative treatments of higher efficiency in removing environmental pollutants as well as recapture otherwise lost resources are of high interest.

It is well known fungi can produce valuable biochemical metabolites and single-cell protein (SCP) on a variety of substrates while simultaneously cleansing the waste [7,8]. Single-cell protein of yeasts and filamentous fungi typically contains all of the necessary amino acids as well as they are easily digested [9]. The potential for SCP production to be used for a feed supplement in fish, broilers and cattle is exceptionally attractive as the amount of food produced today is expected to need to increase by 100-110% by 2050 due to the increasing human population [9,10].

Chemical oxygen demand (COD) is a concentration of organic material that can be chemically oxidized to inorganic products [11]. These organic carbon metabolites are produced by many industrial and domestic wastewater streams from humans and animals. When wastewater streams with a large amount of COD are released into natural waters, stark consequences can occur such as oxygen depletion and as a further result, the dying of fish due to lack of oxygen that was consumed by bacteria [12]. Hence, it is critical that this organic matter is reduced to a level deemed acceptable to be released into the natural environment. It has been observed that filamentous fungal organisms such as *R. oligosporus* and *P. chrysosporium* were able to reduce COD in various wastewater streams by 95% and 74%, respectively on effluents [13,14].

Soybean meal supernatant (SBMS) used in this study is a by-product of the local feed company, which uses microbial processes to upgrade soybean meal into a high-protein animal feed. This process produces a significantly large amount of SBMS and is currently discarded due to the lack of the economically viable methods for its utilization. With this soybean processing supernatant being rich in nutrients and minerals, we hypothesized that the metabolic activity of filamentous fungi may be utilized to achieve the simultaneous reduction in the nutrient loss as well as avoid environmental impacts of this waste stream (due to high chemical oxygen demand). The SBMS which is rich in protein (37.57%, dry basis) and residual carbohydrates would be converted into a more nutritional and highly digestible single-cell protein allowing for a potentially valuable feed ingredient. In this study, eight different fungal strains known to be generally regarded as safe (GRAS) by the FDA were screened to determine an optimal fungal treatment for converting soybean processing waste stream into high-protein animal feed.

Materials and Methods

Wastewater sample

The waste stream of soybean meal (SBM) processing industry was obtained from a local feed company (Prairie Aquatech) in Brookings, SD. The liquid stream of SBM processing industry is referred as SBMS throughout the manuscript. The nutrient composition of the SBMS is shown in Table 1. The SBMS was transferred into closed containers immediately after harvesting and stored in freezer (-20°C) until use. The samples were thawed at room temperature prior to using for experimental trials.

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Components	Percent (m/m)
Moisture content	92.62
Dry matter (DM)	7.38
Crude fiber	1.27
NDF	1.897
ADF	4.39
Crude Fat	N/A
Crude Protein	37.57

Table 1: Proximate composition of SBMS on DM basis.

Organisms	Strain	pH
Aureobasidium pullulans	NRRL-Y-2311-1	3
Trichoderma reesei	NRRL-3653	5
Fusarium venenatum	NRRL-26139	5
Paecilomyces variotti	NRRL-1115	5
Rhizopus microspores var. oligosporus	NRRL-2710	5
Neurospora crassa	NRRL-2332	5
*Mucor circinelloides**	N/A	5
*Pichia kudriavzeii**	N/A	3

*These organisms were contaminants isolated from prior research and identified via 15 sRNA analysis by Kerry O' Donnell's lab (USDA National Center for Agricultural Utilization Research, Peoria, IL, USA)

Table 2: List of fungal strains and optimal pH of the growth media.

Cultures, maintenance and inoculum preparation

Different fungal strains used in this study are listed in Table 2. These strains were provided by the USDA National Center for Agricultural Utilization Research (Peoria, IL, USA). For short-term maintenance, strains were stored on Potato Dextrose Agar (PDA) plates and slants at 4°C. While lyophilization was used for long-term storage of organisms. Seed cultures were prepared in 250 ml Erlenmeyer flasks containing 100 ml of glucose yeast extract (GYE) media (5% glucose and 0.5% yeast extract). The pH of growth media was adjusted for each culture to their optimal pH (Table 2) either by using 10 M sulfuric acid or 10 M sodium hydroxide prior to inoculation. The seed cultures were inoculated by transferring a previously cut square section of agar for filamentous fungi or isolated colonies for yeast-like organisms. Shake flasks were then incubated in a rotary shaker at 150 rpm and at 30°C for 72 h.

Experimental procedures

Lab scale fermentation using 250 ml Erlenmeyer flask: Lab scale fermentation of SBMS was carried out using 8 different fungal strains. Flask trials were performed in 250 ml Erlenmeyer flasks containing 100 ml SBMS. Prior to inoculating with seed culture, SBMS media for each culture was adjusted to their optimal pH and sterilized using an autoclave for 20 min at 121°C. After cooling to room temperature, flasks were inoculated with 2 ml of inoculum of the desired microbe and incubated in rotary shaker at 150 rpm for 5 days at 30°C. After the completion of the incubation period, final pH of the fermented slurry was recorded and samples were harvested using centrifugation at 4,000 rpm for 10 min. Flask contents were then dried in an oven at 80°C for 24 h and total mass was recorded. Both the supernatant and solid fractions were used to determine protein titers. All the experiments were performed in triplicate.

Lab scale fermentation of SBMS using bioflo reactors: The results from flask trials were used to down select the two best performing microbes for bioflo trials. Microbes that produced maximum cell mass and caused significant reduction of solids in the supernatant were chosen to be tested under optimal conditions in a 5 L benchtop reactor (New Brunswick bioflo III, Edison, NJ, USA). Approximately 3 L of SBMS was added to the reactor and was pH adjusted to the optimal for the desired microbe. Reactors were sterilized using an autoclave at 121°C for 30 min. After cooling, reactor settings were fixed at an agitation rate of 250 rpm, 30°C and an aeration rate of 0.8 v/v/min. Upon settings reaching equilibrium, reactors were inoculated with 30 ml of chosen 72 h culture and allowed to grow for 120 h. Daily samples of ~100 ml were collected and used to monitor pH, protein titers, mass balance calculations and reduction of solids in the supernatant. At the end of the incubation period, reactor contents were harvested and dried at 80°C until dry. All the experiments were performed in triplicate.

Analytical methods

Moisture determination: The moisture content of liquid and solid samples were determined using the American Association for Clinical Chemistry (AACC) method [15]. Where ~30 ml and ~0.5-1 g of sample respectively at 80°C for at least 24 h-48 h or at 105°C for at least 4 h.

Total protein: Total protein content of the samples was measured using a LECO model FP528 (St. Joseph, MI, USA). This method uses combustion method to measure the total nitrogen content in the sample. Roughly 0.25 g of sample fractions were placed in a tin foil cups for the combustion. Total protein titers were then calculated by multiplying the total nitrogen content by a conversion factor of 6.25. Total moisture content of the sample was used to determine the protein content on dry basis. All the analysis was duplicated.

Chemical oxygen demand (COD) and solids (X_{TS}, X_{DS} and X_{UDS}): COD was calculated using the closed reflux, titrimetric method APHA 5220c. A total solids fraction (X_{TS}) and a dissolved solids fraction (X_{DS}) were used to calculate an undissolved solids fraction (X_{UDS}). X_{TS} was found by placing the harvested sample in a dryer at 105°C for at least 4 h. X_{DS} was found by centrifuging 10 ml of harvested sample at 4,000 rpm for 10 min and decanting. Then the decanted supernatant was centrifuged at 4,000 rpm for another 10 min and the resulting supernatant was placed in dryer at 105°C for at least 4 h. The dry masses of both the X_{TS} and X_{DS} fractions were used to calculate X_{UDS} using the formula below:

$$X_{UDS} = \frac{X_{TS} - X_{DS}}{1 - X_{DS}}$$

Statistical analysis

All statistical analysis was performed using RStudio 1.0.1.143 [16]. For Erlenmeyer flask trials the Bartlett's test was used to test for homogeneity between variances between treatments [17]. When homogeneity of variances was confirmed, analysis of Variance (ANOVA) was applied to determine the significant differences between fungal treatments in the Erlenmeyer flask trials using the RStudio analysis package 'agricolae' [18]. Significance differences between means were found *post hoc* using Duncan's new multiple range test (MRT) [19].

For 3 L benchtop reactor trials, an F-test was used to confirm the variances were not significantly different between treatments [16]. A two-sample t-test was conducted if variances were not significantly different and a welch two-sample t-test was performed if variances were significantly different [20,21].

Results and Discussion

Proximate composition of the SBMS

The proximate and mineral composition of the SBMS as determined are listed in the Tables 1 and 3, respectively. The SBMS is mainly water (~92.62%), containing only about 7.28% of solid matter on dry basis. This by-product of the soybean processing industry is rich in protein (37.57%, db), fibers (4.39% ADF; 1.89% NDF) and essential minerals

Minerals	Units	Results
Phosphorus	% (m/m)	0.08
Potassium	% (m/m)	0.35
Calcium	% (m/m)	0.03
Magnesium	% (m/m)	0.05
Zinc	PPM	5.22
Manganese	PPM	5.76
Copper	PPM	0.64
Iron	PPM	6.57
Sodium	% (m/m)	0.00
Sulfur	% (m/m)	0.23

% (m/m): Concentration in Percent by Mass; ppm: Parts Per Millions

Table 3: Mineral composition of SBMS on as is basis.

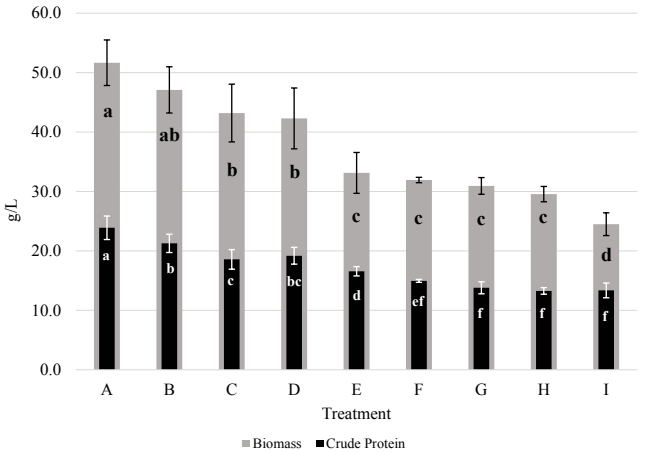
(iron, zinc, manganese, sulfur, and potassium, etc.) and therefore, consisted of a high concentration of chemical oxygen demand (COD). Since the SBMS contains a high COD, is critical to treat the effluent before discharging into the environment

Fungal fermentation using 250 ml Erlenmeyer flasks

The first part of this study consisted of screening of eight fungal strains on the SBMS. After the 5 days incubation period, all fungi except for *P. kudriavzevii* produced significantly higher (P<0.05) amounts of biomass than the control. The respective biomass concentrations for the top performing strains, i.e., *T. reesei, N. crassa, F. venenatum* and *P. variotii* were 51.7, 47.1, 43.2 and 42.3 g/L. Whereas, the rest of the fungal treatments yielded between 29.6-33.1 g/L biomass (Figure 1).

As explained in the above materials and methodology section, after the completion of the fermentation cycle, the solid and liquid fractions were separated using centrifugation and each fraction was then subjected to protein analysis. The protein titer of the solid fraction of all the treatments, including control is listed in the Table 4. Protein titers analyzed on the treated product varied from 41.7-53.6% (Table 4) whereas, the uninoculated control contained ~54.5% protein. Among the various strains evaluated, *P. kudriavzevii* yielded the highest protein titer in solid fraction, similar to that of un-inoculated control (~53.6% *vs.* 54.5%; Table 4). However, the protein titers in the rest of the fungal treated solid fractions were significantly lower than that of the control. This could probably be explained by the fact that the extracellular enzymes produced by the fungi would lead to the degradation of the longer peptides into highly soluble form of protein, which would ultimately be lost in the supernatant during separation. Therefore, protein titers in the supernatant fractions were also determined and results showed that only with the exception of *T. reesei, P. kudriavzevii* and *A. pullulans*, all other fungal treatment resulted in the supernatant with high protein titers as compared to the control (data not shown).

To fully understand the effectiveness of the overall process, mass balance calculations were conducted to determine protein and biomass yields (Figure 1). As shown in Figure 1, despite of being high in protein titers, the untreated control resulted in the lowest biomass and protein yield when compared to that of any fungal treatment. The protein yields for various fungal treatments ranged from 13.3-23.9 g/L and the maximum yields were obtained by the *T. reesei* and *P. variotii* treatments at 23.9 and 21.3 g/L, respectively (Figure 1). Likewise, *N. crassa* and *F. venenatum* also resulted in crude protein yields similar to that of *T. reesei* and *P. variotii* hence, improvement in the protein yields were achieved (Figure 1). The total amount of supernatant solids reduced due to the fungal fermentation process was determined by subtracting the solids present in the supernatant fraction at the end of the fermentation from the supernatant solids present in the un-



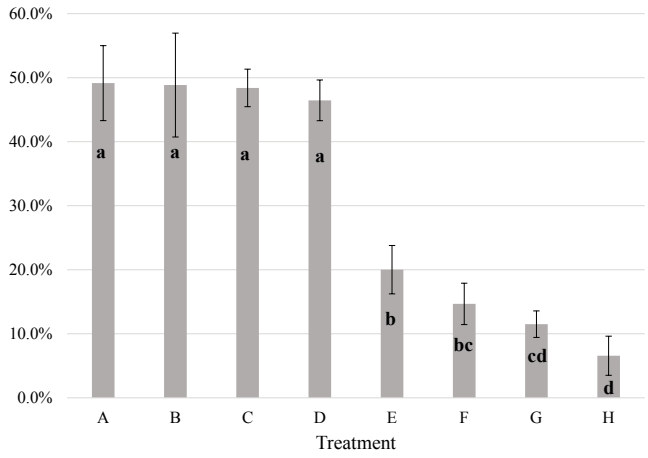
Mean value sharing the same letters are not significantly different from each other (p<0.05)

Figure 1: Biomass and Crude Protein yields after 5 d treatments. (A) *T. reesei* (B) *P. variotii* (C) *N. crassa* (D) *F. venenatum* (E) *R. oligosporus* (F) *M. circinelloides* (G) *P. kudriavzevii* (H) *A. pullulans* (I) Uninoculated control.

Treatment	Protein Titer (%)
Uninoculated Control	54.5 ± 1.0^{a}
P. kudriavzevii	53.6 ± 1.0^{a}
M. circinelloides	46.8 ± 0.6^{b}
T. reesei	46.3 ± 1.6^{b}
F. venenatum	45.6 ± 2.5^{bc}
P. variotii	45.2 ± 0.5^{bc}
A. pullulans	44.9 ± 0.6^{bc}
N. crassa	43.1 ± 2.1^{cd}
R. oligosporus	41.7 ± 2.6^{d}

Mean value sharing the same superscript letters are not significantly different (p<0.05)

Table 4: Protein titers after 5 days incubation in Erlenmeyer flask trials.



Mean value sharing the same letters are not significantly different from each other (p<0.05)

Figure 2: Reduction in supernatant solids in flask trials. Values obtained by comparing to uninoculated control. (A) *N. crassa* (B) *P. variotii* (C) *F. venenatum* (D) *T. reesei* (E) *R. oligosporus* (F) *A. pullulans* (G) *M. circinelloides* (H) *P. kudriavzevii.*

inoculated control. Percent solid reduction due to fungal treatment ranged from 6.6-49.1% (Figure 2). Among all the fungal treatments, the

maximum solid reduction of 49.1, 48.9, 48.4 and 46.5% were achieved, respectively, with *N. crassa, P. variotii, F. venenatum* and *T. reesei* (Figure 2). This study clearly indicated that among the eight strains that were evaluated, *T. reesei, N. crassa, F. venenatum* and *P. variotii* showed high potential for converting SBMS into protein rich feed. Previously, Simon et al. [22] also reported the maximum protein yield on carinata meal using *N. crassa, P. variotii* and *T. reesei*. From this study, T. *reesei* and *N. crassa* were chosen for the benchtop reactor trials for high biomass and corresponding protein yields as well as extremely high growth rate on the SBMS media in the case of *N. crassa*. The other two strains (*P. variotii* and *F. venenatum*) were excluded from the benchtop reactor trials as they visibly grew at a slower pace during the seed culture phase. Additionally, a literature review found instances of allergic reactions to *F. venenatum* protein [22].

Benchtop reactor trials using *T. reesei* and *N. crassa*

Benchtop reactor trials conducted with the down selected treatments of *T. reesei* and *N. crassa* resulted in biomass yields of 55.5 and 62.0 g/L after 5 days incubation under optimal conditions (Table 5). The protein titers in the solids fraction of both trials (benchtop trials and flask trials) using *T. reesei* and *N. crassa* was consistent with *T. reesei* having ~3% higher titers in either scenario (Tables 4 and 5). However, an increase in biomass yield was observed for the benchtop trials when compared to the initial flask screening trials. This improvement in biomass yield could be attributed to the aeration and agitation provided by the fermenter. *N. crassa* obtained higher and statistically significant ($p<0.05$) yields of 27.3-28.5 and 61.1-63.2 g/L of crude protein and biomass respectively despite a slightly lower protein titer compared to *T. reesei* (Table 5). Yields and protein titers conducted in benchtop reactors were comparable to flask trials.

Treatment	Biomass Yield (g/L)	Protein Titer %	Protein Yield (g/L)
N. crassa	62.0 ± 1.1	45.0 ± 0.8%	27.9 ± 0.6
T .reesei	55.5 ± 1.5	48.1 ± 1.2%	26.7 ± 0.2

Table 5: Biomass after 5 days incubation in benchtop reactors with 3 L total volume.

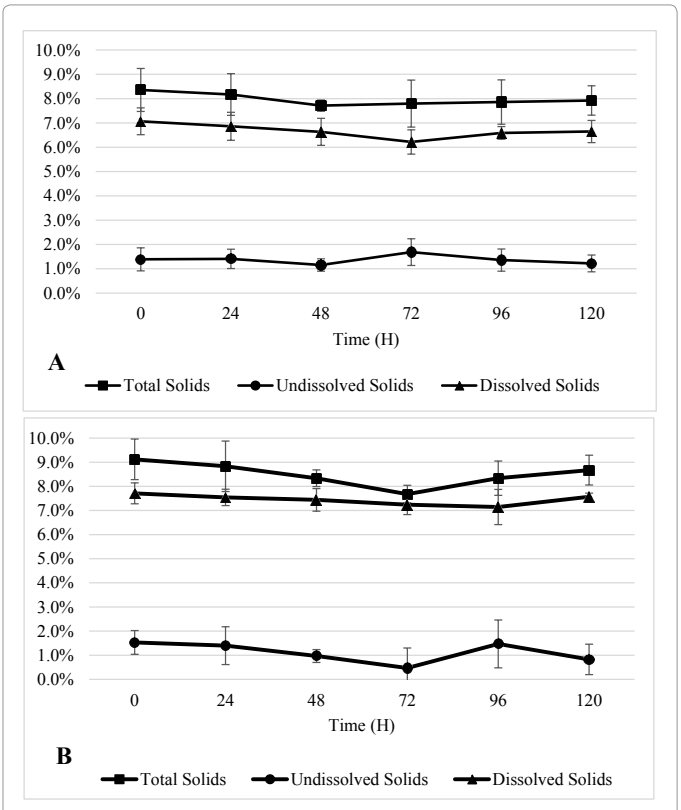


Figure 3: Total solids, undissolved solids and dissolved solids over the course of the 5 days incubation in benchtop reactors with 3 L total volume treated with (A) *N. crassa* (B) *T. reesei*.

The mean X_{TS} for either treatment began around 8.36-9.12% (Figure 3) even though the material contained ~7.4% dry matter. Autoclaving the material resulted in a slight loss of (~300 ml) SBMS from the reactor which may have concentrated the amount of solids in the reactor. Throughout the incubation, *N. crassa* and *T. reesei* reduced mean X_{TS} in the SBMS by 0.57% and 0.79%, respectively. Differences in which solids were apparently utilized by each organism were seen between the two treatments. Mean X_{UDS} remained unchanged throughout the study when treated with *N. crassa,* while mean X_{DS} was reduced from 7.1 to 6.7%. On the contrary, *T. reesei* reduced mean X_{UDS} from 1.5 to 0.8% and mean X_{DS} from 7.7 to 7.6%. Thus, it appears *N. crassa* was more effective in reducing dissolved solids while *T. reesei* was more effective in reducing undissolved solids.

The pH value of the SBMS dropped slightly after autoclaving. Throughout the fermentation, the pH steadily rose. *N. crassa* obtained a pH value of 6.53-6.88 throughout the incubation while *T. reesei* obtained a pH value of 6.08-6.40 (Figures 4 and 5). *N. crassa* and *T. reesei* are both known to produce extracellular enzymes capable of breaking down the

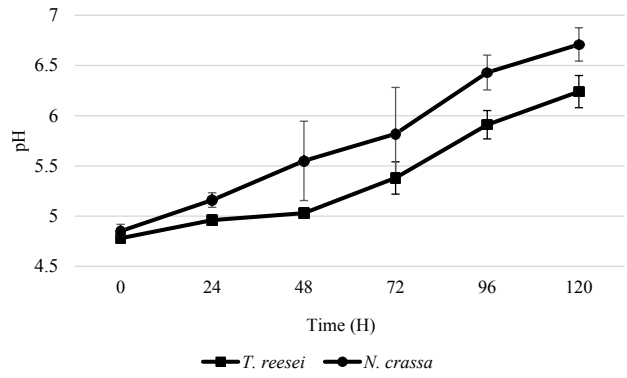


Figure 4: Relationship between pH and time of SBMS while treated in benchtop reactors with 3 L total volume.

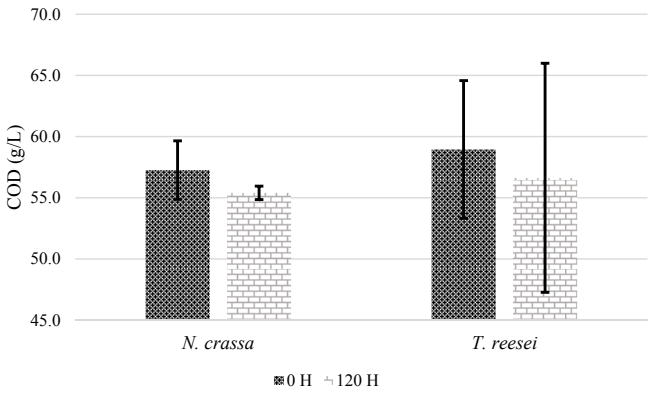


Figure 5: COD measurements after 5 days treatment of SBMS.

peptides. Hence, the production of the alkyl groups (NH_3, NH_4) during fermentation may have led to this slight increase in pH.

Change in COD level of SBMS after fungal fermentation using *T. reesei* and *N. crassa*

Using fungal fermentation to reduce the COD level of the wastewater from industrial processing has been studied previously [6,23]. SBMS used in this study was rich in protein resulting in COD measured at 57.64 g/L. We found that *N. crassa* and *T. reesei* reduced the COD level by 23.04% and 10.53%, respectively after 5 d of incubation. This reduction in COD level would probably be improved further by lengthening the fermentation process. For example, a previous study reported that a longer incubation time (21 days after inoculation) proved to be beneficial in decreasing the amount of organic matter in the supernatant [24]. At the same time, there are studies reporting more than 90% of COD removal in less than a 24 h of inoculation. For instance, a starch processing wastewater treated with *R. oligosporus* NRRL-2710 reduced the COD by over 95% in just 16 h. Initial COD levels of the starch processing wastewater were 11.9 g/L to 18.9 g/L [13]. An additional study using olive mill wastewater found that COD values of 55-60 g/L inhibited the growth of *P. chrysosporium* and resulted in no decolorization activity due to the presence of a large amount of high-molecular weight aromatics [25]. Thus, the findings from the literatures and the results obtained in our study led us to conclude that there could be various parameters which would affect the COD reduction of the waste stream. Hence, further studies should be conducted focusing on optimizing process parameters to achieve adequate reduction of COD levels.

Conclusion

Fungal incubation treatments produced protein-rich biomass for potential use in animal feed while simultaneously reducing various solids in the supernatant fraction of the soybean processing wastewater. *N. crassa* and *T. reesei* were down-selected for bioreactor trials as they outperformed other fungal strains tested in flask trials. *N. crassa* and *T. reesei* produced 62.0 and 55.5 g/L of biomass after 5 days incubation with 44.2-45.8% and 46.9-49.3% of that biomass existing as crude protein respectively. This study elucidated an interesting possibility of simultaneously producing protein-rich animal feed ingredient and reducing organic components in the SBMS by using fungal metabolism.

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