HIGH-YIELD PROTEIN RECOVERY FROM SECONDARY SLUDGE OF PAPER MILL EFFLUENT AND ITS CHARACTERIZATION

Muhammad Pervaiz* and Mohini Sain

Maximizing recovery and characterization of extractable proteins from secondary paper sludge is essential to explore the potential value from utilization of readily available waste products from pulp and paper mills. A multistep physicochemical recovery process was used, involving optimum alkaline solubilization of protein into an aqueous phase followed by augmented physical disruption of cell membranes. The final precipitation of solubilised protein was carried out using different acidic media. The optimization studies revealed that the best removal of intercellular contents from sludge can be achieved at pH 12; at this level, up to 88% of available protein is solubilised into the aqueous phase. Of all the precipitating agents used, sulphuric acid proved most effective by recovering 90% of disrupted protein. The combined effect of french press and sonication techniques resulted in significant improvement in the overall yield of recovered sludge protein (RSP). The characterization studies showed the presence of common and essential amino acids in RSP in significant quantities; it also showed that the recovery process can significantly reduce or eliminate heavy metals present in the sludge. The molecular weights (MW) of extractable proteins were determined by PAGE, and it was observed that RSP contains both low and high MW fractions.

Keywords: Recycling; Secondary sludge; Protein recovery; Physicochemical treatment; Protein characterization; Residual biomass; Amino acid; Metal toxicity

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INTRODUCTION

Pulp and paper manufacturing facilities generate approximately 45 kg of waste sludge per ton of product, depending on the nature of raw materials (Edalatmanesh *et al.* 2010). The global quantity of paper mill biosolids generated annually is increasing rapidly, and it is estimated that it will reach up to 4.5 million tons by the year 2050 (Rashid *et al.* 2006). This huge quantity of waste biomass has consistently posed serious challenges for the paper industry, requiring extra economic resources to deal with disposal and environmental issues.

A considerable portion of the nitrogenous materials present in the sewage of paper mill effluents is settled out during the treatment processes and is converted into primary sludge through the sedimentation process. Alternatively, the nitrogenous material may be removed from the sewage effluent and be converted into the biomass of microorganisms through the biological oxidation and coagulation processes known as the activated secondary treatment process (Lau 1981). The activated sludge process generates a large amount of excess sludge due to consumption of organic pollutants in the wastewater and the associated microbial growth (Jung *et al.* 2001).

So far, excess sludge is mainly handled through soil application, combustion, landfill, or ocean dumping. Due to the high costs of excess sludge treatment, around 50% of which is associated with effluent treatment cost (Zhang *et al.* 2007), and scarcity of disposal lands, it has become imperative to develop new technologies in finding novel uses for this residual biomass, which is rich in valuable components.

Secondary sludge (SS), generated through biological treatment of effluent, typically consists of polysaccharides, nucleic acids, enzymes, and proteins (Garcia *et al.* 2010). The characteristics of secondary sludge compared to other common sludge types are shown in Table 1.

Table 1. Comparative Analysis of Various Types of Common Sludges

Property	SS[1]	MS[1]	PS[2,3]	DPS[2]	Sew. S[4]
Ash	23.7	30.2	19.5	53.5	17.9
Lipid	3.7	0.3	-	-	15.6
Protein	26.8	7.9	-	-	34.9
Carbohydrate	10.1	39.8	62.7	4.8	22.2
Klason Lignin	20.2	28.6	27.8	24.9	11.3

SS: secondary sludge, MS: mixed sludge, PS: primary sludge
DPS: de-inked paper sludge, Sew. S: sewage sludge
1. Current study
2. Geng *et al.* 2007
3. Deng *et al.* 2007
4. Barzelatto 1995

Since bacterial cells are believed to contain about 50% protein (Shier and Purwono 1994), the SS provides an excellent opportunity to be explored as a source of proteinaceous substances. The potential for reutilizing SS as a resource is possible either by directly recovering useful biomaterials or modifying SS into value-added materials through biological or physicochemical techniques. So far the crude protein from activated sludge has been used on a trial basis as an alternate animal feed and biobased wood adhesive (Hwang *et al.* 2008; Pervaiz and Sain 2010).

Earlier research studies have mentioned the recovery of protein from sewage treatment plants, wastewaters of fish processing facilities (Stine *et al.* 2012), cheese manufacturing, and poultry industries (Potter *et al.* 1974). Though literature is not very specific about the true optimum conditions necessary to obtain maximum protein extraction, various protein recovery protocols based on physicochemical techniques have been used, some of which start with solubilization of intracellular sludge contents into the aqueous phase by disrupting the floc structure (Jung *et al.* 2001; Onyeche *et al.* 2002).

Unfortunately, most protein recovery efforts have focused only on sewage sludge of urban municipalities (Lerch *et al.* 1993a), extracting the proteins from sewage sludge by using H_2O, Triton X·l00 (a non-ionic detergent), and 1M NaOH. It was reported that 1M NaOH was about 4.6 times more effective than a detergent in solubilizing the proteins, whereas H_2O was only half as effective as the detergent. Lau (1981) has reported protein recovery from both primary and secondary sludge of three different sewage plants by using an ion-exchange process followed by 2.5% sodium chloride solution to facilitate protein precipitation. Finally, sodium lignosulphonate was used as the precipitating agent. The efficiency of protein extraction into solution from sludge is reported to be around l0%, while protein recovery from the solution using sodium lignosulphonate was approximately 38%. The recovered sludge protein had considerable

protein content: as high as 71% for the secondary sludge. Stafford *et al.* (1979) employed an extraction process using hot alkali as the solubilizing agent and obtained a yield varying between 30 to 45% of the original sewage sludge on dry weight basis. Protein recovery from poultry processing wastewater using membrane ultrafiltration has been also studied, whereby up to 3.4 times enrichment in protein concentration compared to initial solubilized protein is reported (Lo *et al.* 2005)

In our previous study (Pervaiz and Sain 2010), paper mill SS was used for the first time as a resource to extract protein, employing alkali treatment followed by low-pH centrifugation. In the current study a combination of french cell press and sonication was used to increase disruption of floc structure in sludge solids. These methods have been used by researchers for cell lysis (Benov and Al-Ibraheem 2002; Abram *et al.* 2009). The present research incorporated these techniques in addition to the traditional alkali solubilization process in order to increase the yield of protein extraction from paper mill SS.

The precipitation of soluble protein in the present study was evaluated using sulphuric acid, hydrochloric acid, and ammonium sulphate. The extracted crude protein was characterized for quantitation, MW determinations, amino acid evaluation, and assessment of heavy metal toxicity. The results of RSP characterization were compared with available data pertaining to sewage sludge protein leading to a conclusive discussion in exploring the potential utilization of paper mill SS.

EXPERIMENTAL

Materials and Methods

Sample collection

Activated secondary sludge in liquid form was obtained from a Canadian paper mill located in Ontario, in polyethylene bags, and stored at 4 ^{o}C until testing. Reagent grade alkali (sodium hydroxide), acid (sulphuric and hydrochloric), and ammonium sulphate were used for protein solubilization and precipitation studies.

Preliminary characterization

The solid contents of liquid SS were determined by standard method (APHA 2005). For the rest of preliminary characterization, SS was dried at 60 ^{o}C until constant weight. Next the dried materials were ground in a Wiley mill fitted with a 40-mesh screen.

The ground material was used for further tests. The ash content was tested using TAPPI Test Method T 211 om-07. The lipid fraction of SS was estimated in a standard Soxhlet apparatus in which toluene was the solvent and lipid content was measured by loss in sample weight. Klason lignin content was estimated according to TAPPI Test Method T 222 om-88.

Protein recovery

A physicochemical procedure was used to recover protein from SS as shown in the schematic of Fig. 1. Each step of protein recovery and characterization details are mentioned below.

Solubilization of intracellular materials

The initial solubilization of intercellular sludge contents into the aqueous phase was done by alkali treatment using liquid SS as the starting material containing 2.6% solids. Since sodium hydroxide is reported as the most effective solubilising agent (Chishti *et al.* 1992; Lerch *et al.* 1993a; Hwang 2008), it was used to disintegrate and release the intracellular materials into the aqueous phase. Various pH values ranging from 8.0 to 12.5 were maintained by treating with alkali at 25 ^{o}C, followed by continuous stirring at two different durations, 2 and 24 hours. Finally, protein concentration was determined using the Bradford assay method in alkali treated sludge solutions to calculate solubilization yield at each pH level.

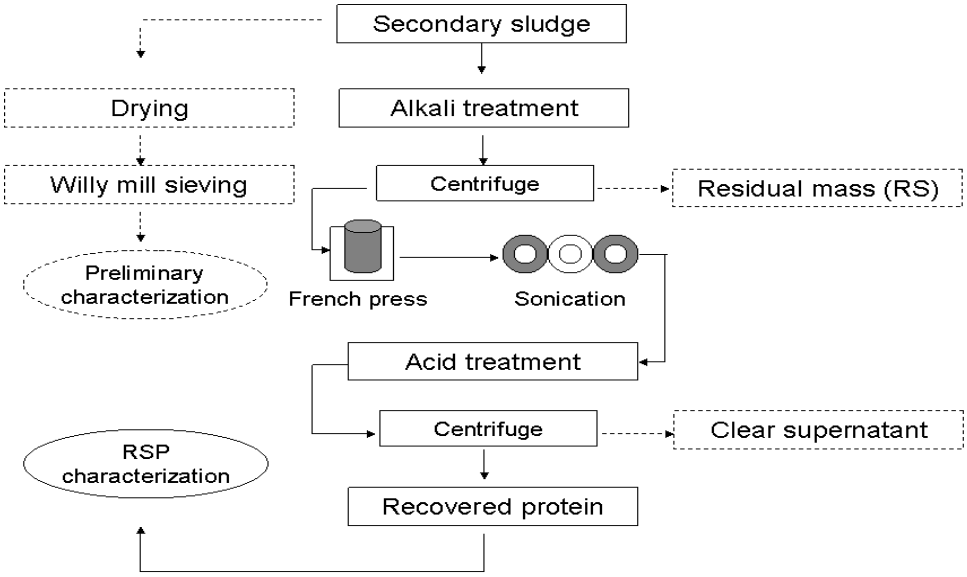

Fig. 1. Schematic of protein recovery protocol

Cell disruption

Cell disruption was done on a SLM Aminico® French Pressure Cell (40 mL), *Spectronics Instruments-USA*, by adjusting the pressure to 20,000 psi. A quantity of 30 mL of alkali treated (12 pH, 24 h treatment) sludge was poured into a minicell for each pressing, and six replications were carried out for each test.

Sonication was performed on a Sonifier® 450 (400 Watts, 60Hz), *Branson Ultrasonics Corporation-USA*, equipped with a micro tip. French pressed sludge samples were intermittently sonicated on ice for 30 seconds with 30 seconds allowed for cooling. The total sonication time was 1, 3, or 5 minutes. Six replicates were processed for each cycle.

The total number of French press cycles and duration of sonication were varied in this study to investigate the maximum release of proteins from alkali treated sludge.

The disrupted floc mass containing mostly soluble protein was separated as supernatant by centrifugation of the disintegrated sludge at 7000 rpm for 30 minutes at 4 ^{o}C.

Protein precipitation

In previous studies (Pervaiz and Sain 2010), optimized precipitation of soluble protein was achieved at a pH value of 3.0. In the present study the soluble protein from

the supernatant after physicochemical treatment was recovered using three different precipitating agents, separately.

2.0 M solutions of reagent grade sulphuric and hydrochloric acids were used to lower the pH of the supernatant to 3.0, and the precipitates were centrifuged at 7000 rpm for 30 minutes at 4 C^o to obtain RSP in the pellet form.

A 40% saturated solution of ammonium sulphate was used to precipitate protein from the protein solution. To remove excess salt from the recovered protein, dialysis was performed using regenerated cellulose dialysis tubes (Fisherbrand #21-152-5).

The final pellet of RSP in each case was dried at 60 C^o overnight to estimate yield and for use in various characterization studies.

Characterization

Protein estimation

All soluble protein measurements were done by the Bradford method (Bradford 1976) using γ-globulin standard solution. Total protein in SS and RSP was estimated by first determining the total organic nitrogen through Kjeldahl method, as described in Standard Methods for the Examination of Water and Wastewater (APHA 2005), and then multiplying the resultant value by 6.25.

Metals

The concentrations of heavy metals in SS and RSP were determined using an inductively coupled plasma mass spectrometer (Perkin Elmer Model Optima 7300DV ICP AEOS-Perkin Elmer Inc., USA). In short, the dried and powdered samples were acid digested, diluted volumetrically with 18 Mohm water, and assayed by ICP AES directly.

Amino acid analysis

Precipitated protein using H_2SO_4 was analysed to determine amino acid composition of RSP. The method involved hydrolysis using 6 N HCl and pre-column derivatization of the hydrolyzates using phenyisothiocyanate (PITC) followed by reverse phase HPLC. The reverse phase HPLC method is a common technique to determine amino acid concentrations of proteinacous materials (Chishti *et al.* 1992)

SDS-PAGE analysis

The lysis of samples for extraction of protein from the sludge slurry was performed by treatment with alkali and liquid nitrogen, with or without detergents (NP-40, Triton X-100, or a combination of these surfactants). To prevent endogenous protease activity, protease inhibitor cocktails were also added to the sample. After cell lysis and inactivation of interfering substances, some samples proteins were also solubilized with 7 M urea. Unless otherwise stated, samples were boiled for 5 minutes in sample buffer (final concentration 0.25 M Tris-HCl, pH 6.81, 30% (v/v) glycerol, 8% (w/v) SDS) supplemented with 10% (v/v) 2-mercaptoethanol, bromphenol blue indicator dye (Sigma chemical) and centrifuged to remove any un-dissolved material. The loaded sample volume for each well of gel was 20 µL, whereas for the marker, a pre-stained SDS-PAGE standard broad range ladder (Bio-Rad: 161-0318) was used.

The SDS-PAGE procedure was routinely carried out using 4 to 20% precast linear gradient polyacrylamide gel, 10-well, 30 µL, 8.6 x 6.8 cm (W x L), while using a Mini-Protein II Bio-Rad electrophoresis system working on an electrical potential of 120 V and

60 mA. Proteins were stained using brilliant blue G solution (Sigma B8522-1EA) containing 0.1% w/v brilliant blue, 25% v/v methanol, and 5%v/v acetic acid.

Fourier transform infrared (FT-IR) analysis

The study on the functional groups of secondary sludge, recovered protein, and residual mass was performed on a Bruker Tensor-27 spectrometer. All spectra were captured over a range of 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1} with 200 scans.

RESULTS AND DISCUSSION

Protein Solubilization

Since most of the proteins in activated sludge are not in solution form, it cannot be readily separated from other complex organic and inorganic materials. It is reported (Sridhar and Pillai 1973; Christiansen and Mitchell 1978) that chemical treatment involving alkali is a suitable solubilization method by which encrusting substances (cellulose, hemicellulose, and lignin) are effectively removed. The effect of sodium hydroxide on paper mill secondary sludge was studied at different pH levels for two different time intervals. A linear increase in protein recovery was observed with increase of hydroxyl ions for both the 2 and 24 hour intervals, as shown in Fig. 2. The maximum protein recovery was possible at pH 12 for both time intervals, beyond which no improvement in solubilization was observed. However, after pH 10 the increase in recovery rate was steeper for the 24 hour treatment in comparison to the 2 hour run. The maximum recovery of available protein was achieved at pH 12.0 (24 hour treatment), which was about 32% more compared to the 2 hour treatment at the same pH. A high quantity of alkali was required to increase the pH from 12 to 12.5, and a 3 to 4% drop in protein recovery was observed for the same incremental shift in pH level.

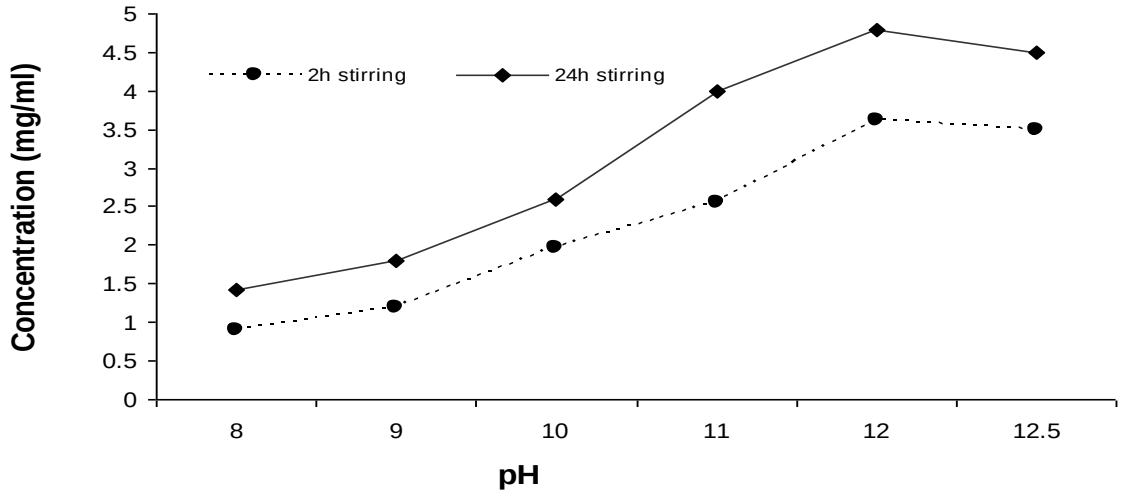

Fig. 2. Effect of pH and reaction time on protein solubilization

Cell Disruption (French Press and Sonication)

To augment the protein yield after alkali treatment, optimization studies were carried out using the French press and sonication methods individually as well as in combination where French pressing was followed by sonication.

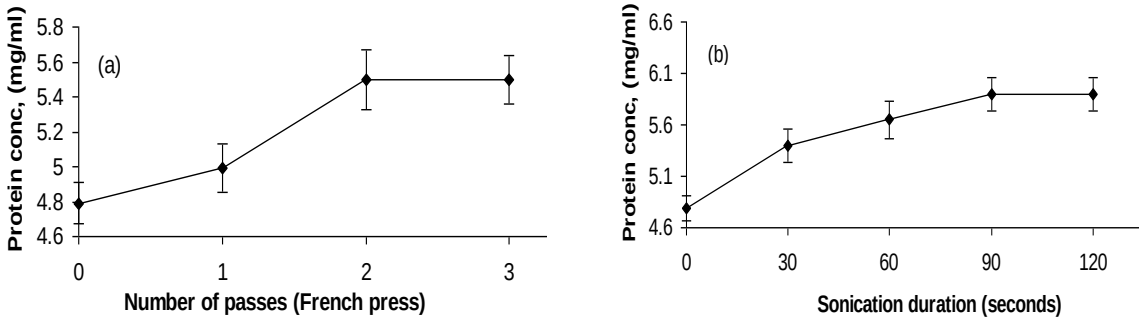

Fig. 3. Protein yield optimization; effect of number of passes (French press) and sonication time on the solubilization of alkali treated sludge protein

In stand-alone mode, alkali treated sludge was used as the starting material individually for both French press and sonication studies. The effects of the number of passes in French press and duration of sonication are shown in Fig. 3. After two passes of French press, a maximum cell disruption was observed, showing a 15% increase in protein solubilization compared to the alkali treated control sample. After two passes, no improvement was noticed, which agrees with previous research (Benov and Al Ibraheem 2002). Sonication was a better choice, which yielded 23% more solubilised protein compared to the alkali-treated control sample. The maximum yield was obtained in about 3 to 4 sonication cycles of 30 seconds each, beyond which no further solubilization was observed.

To investigate the combined effect of French press and sonication, the samples from French press having the maximum solubilised protein were further processed through sonication for a total of four cycles. The results shown Fig. 4 illustrate that an additional protein solubilization of 1mg/mL can be achieved with a combined approach; thereby enhancing the overall protein yield by 44% compared to alkali treated SS.

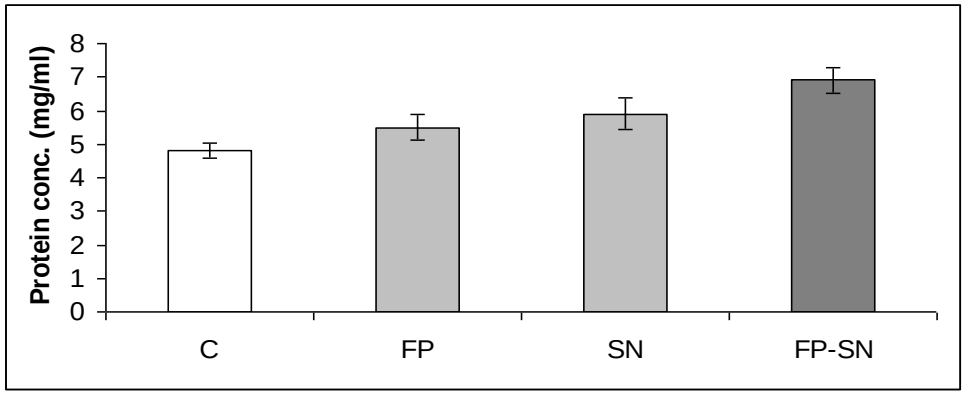

Fig. 4. Combined effect of French pressing and sonication on protein solubilization. (C: control; alkali treated sludge, FP: French pressing, SN: sonication, FP-SN: French pressing followed by sonication)

Protein Precipitation

Procedures involving sufficient amounts of acids, inorganic salts, or organic solvents have been used in the past to extract protein from solubilized protein solutions of different origins (Florkin and Stoz 1963; Sastry and Virupaksha 1967; Hwang *et al.* 2008). In this study instead of using organic solvents as precipitating agents, commonly available dilute acids and inorganic salt were used to recover protein from solubilized protein solutions processed from paper mill SS. The commonly used precipitating agents mentioned in literature are H_2SO_4, HCl, and $(NH_4)_2SO_4$ (Knorr *et al.* 1977; Christiansen and Mitchell 1978).

Table 2. Effect of Different Precipitating Agents on Protein Recovery

Reagent	Quantity Used[1]	Dry wt. of precipitates (g/L)	Protein content (%)	Protein Recovery (%)
H_2SO_4	8.0 mL/L (±0.32)	12.7 (±0.49)	49.0 (±2.30)	90.1
HCl	31.4 mL/L (±1.63)	11.2 (±0.50)	44.6 (±2.20)	72.4
$(NH_4)_2SO_4$	40% sat. soln.	8.9 (±0.43)	46.3 (±2.31)	59.7

All values mean of three replicates

1. Quantity of precipitating agent used to bring down the pH of protein solution from 12 to 3.0
2. 40% saturated solution of $(NH_4)_2SO_4$

Maximum recovery of solubilized protein, around 90%, was achieved using H_2SO_4 at pH 3.0, followed by HCl and $(NH_4)_2SO_4$, as shown in Table 2. Protein content of $(NH_4)_2SO_4$ treated precipitates was higher than HCl treated precipitates; however the protein recovery was less for $(NH_4)_2SO_4$ due to the low amount of centrifuged mass. The results clearly indicate that most of the protein from solution can be extracted using these precipitating agents. The most cost-effective method proved to be H_2SO_4 treatment in terms of yield and ease of operation. The $(NH_4)_2SO_4$ treatment required more time due to the added steps of membrane dialysis to remove residual salt from recovered protein precipitates. This extra filtration step was detrimental for process efficiency and yield due to inherent material losses.

Protein precipitated using H_2SO_4 was used for all the characterization studies.

Metal Toxicity

The measured concentrations of hazardous trace elements including heavy metals in the original activated sludge and the RSP pellet are listed in Table 3 along with some literature values.

The starting sludge contained a variety of metals, which largely depended on the type of raw materials and additives used for pulp and paper manufacturing. Significant amounts of metal were reduced during the protein recovery process, as seen in Table 3. In some cases the concentration of heavy metals was reduced below detection (Cd, Ni, Pb), while in another instance it was reduced by 4 to 6 times the original value (Cu, Fe, Zn). The only exception was Na, which showed an increase after protein recovery; this can be attributed to use of NaOH for solubilization purposes. The low concentration of metals in DIP sludge, as compared to virgin wood, is attributable to cleaner furnish and recycled paper. Overall, paper SS has less metal toxicity compared to municipal sludge except in Ca levels, which pertains to extensive use of calcium carbonate in paper manufacturing. Recovered protein from sewage sludge is also shown (Hwang *et al.* 2008) as lower in

metal concentration compared to its original sludge; however the recovery process is not as effective in reducing metal toxicity, as also demonstrated by the current study. Compared to composting and poultry feed standards, the recovered protein from paper sludge is definitely safe for animal feeds and other value-added utilizations such as wood adhesives.

Table 3. Trace Element Concentrations (%age) in Raw Sludge and Recovered Proteins from Different Sources and Compost and Dairy Feed Standards

Element	Biomass Type						Canadian compost standard[2]. (class B)	Poultry feed legal limit[4]
	SS[1] (Paper)	Rec, protein (SS[1])	DIP sludge[2]	Muncipal Sewage				
				PS[3]	SS[4]	Rec. protein (SS[4])		
Al	1.190	0.311			0.776	0.464		
As	BDL	BDL	<0.010				0.075	$2x10^{-4}$
B	0.004	0.001	<0.001					
Ca	0.774	0.164			0.173	0.091		
Cd	0.001	BDL	$<1x10^{-4}$	0.002			0.002	$1x10^{-4}$
Cr	0.003	0.002					0.106	0.01
Cu	0.048	0.008	0.011	0.094	0.278	0.025	0.076	
Fe	0.571	0.111	0.043		0.504	0.288		
K	0.137	0.073						
Mg	0.339	0.022						
Mn	0.023	0.006	0.002	0.016				
Mo	BDL	BDL					0.002	
Na	0.950	1.940						
Ni	0.003	0.001	<0.001	0.015	0.005	0.001	0.018	
Pb	0.056	BDL	<0.002	0.053	0.002	0.000	0.050	0.001
Ti	0.015	0.003						
Zn	0.080	0.024	0.004	1.38	0.058	0.021	0.185	

SS: Secondary sludge, PS: Primary sludge, DIP: De-inked pulp sludge, BDL: Below detection limit

1. *Current study*
2. *Beauchamp et al. 2002*
3. *Chishti et al 1992*
4. *Hwang et al. 2008*

Amino Acids

A detailed analysis of the amino acid composition of protein extracted from paper SS was carried out, and the results are shown in Table 4, which also includes similar data of other protein products for comparison. This quantitative analysis explicitly indicates the value of sludge protein in terms of amino acid contents, especially the essential amino acids whose levels exceeded those recommended by the Food and Agricultural Organisation (FAO), making the paper sludge an attractive candidate to be explored for food supplements. The results further indicate that the recovered protein is compatible with proteins recovered from sludge of municipal sewage treatment plants in terms of amino acid contents. Another finding is the close similarity between soy flour and paper sludge proteins in terms of amino acid composition. Since soy flour is the most common raw material for producing soy protein wood adhesives, these results show a possibility of replacing food crops with abundantly available biomass from paper mill sludge to develop bio-based wood adhesives.

SDS-PAGE

The composition of RSP was analyzed by SDS-PAGE, Fig. 5. The most distinct patterns were obtained by using a combination of liquid nitrogen and detergent or liquid nitrogen, detergent, and protease inhibitor (Sample A and B). The SDS-PAGE shows that activated SS contained proteins in two distinct MW ranges; the higher MW proteins were found concentrated in the range of 30 kDa to 70 kDa. The other band (not very distinct) of proteins in paper sludge had low MW and was mostly concentrated around 7 kDA.

Table 4. A Comparison of Amino Acid % Composition of Recovered Protein from Paper Mill Sludge and Other Sources

Amino Acid	Recovered Protein			Protein products		FAO[5]
	Paper mill sludge[1]	Muncipal sewage sludge[2]	Muncipal sewage sludge[3]	Soybean flour[4]	Wheat flour[2]	
Asparagine	12.1	-	2.3[6]	11.3	-	
Glutamine	10.8	-	3.3[7]	17.2	-	
Leucine*	8.7	6.9	6.2[8]	6.5	7.0	4.8
Alanine	7.5	-	2.3	4.0	-	
Valine*	7.2	5.4	3.3	4.6	4.1	4.2
Arginine	7.0	-	1.6	7.0	-	
Phenylalanine*	5.9	4.2	2.2	4.7	5.5	2.8
Glycine	5.8	-	2.2	4.0	-	
Threonine*	5.6	5.4	1.6	4.3	2.7	2.8
Tyrosine*	5.5	3.1	1.3	3.4	-	1.4
Isoleucine*	5.5	-	-	4.8	4.2	4.2
Proline	4.7	-	1.4	4.7	-	
Lysine*	4.5	9.0	2.4	5.7	1.9	4.2
Serine	4.2	-	0.8	5.0	-	
Histidine	2.4	-	1.6	2.6	-	
Methionine*	2.4	4.6	0.5	1.3	1.5	2.2
Cysteine*	0.1	1.0	-	1.5	1.9	2.0

1. Recovered protein from secondary sludge of paper mill effluent; current study
2. *Lau 1981*. Recovered protein from secondary sludge of municipal sewage.
3. *Chishti et al. 1992*. Recovered protein from primary sludge of municipal sewage
4. *Cheng 2004*.
5. Provisional amino acid requirements for food products, recommended by Food and Agricultural Organisation (Lau 1981)
6. Aspartic acid
7. Glutamic acid
8. Includes both Leucine and Isoleucine

* Essential amino acids

There is no literature available on SDS-PAGE analysis of paper SS; however Lerch *et al.* (1993b) have reported high MW (29 to 66 kDa) and low MW fractions (< 17

kDa) of proteins extracted from activated sludge samples of different sewage treatment plants by using water or alkali as solubilizing agents.

Goodwin and Forster (1989) have reported the presence of predominantly low MW proteins < 10 kDa, in activated sludge while using membrane filtration. However, in another study the major MW fraction of protein and nonprotein components from activated sludge has been reported as greater than 5 kDa (Karapanagiotis *et al.* 1989). Alkali extraction is most likely responsible for hydrolysis of peptide bonds, generating lower MW components, which is supported by earlier studies (Zubay 1983; Lerch 1991).

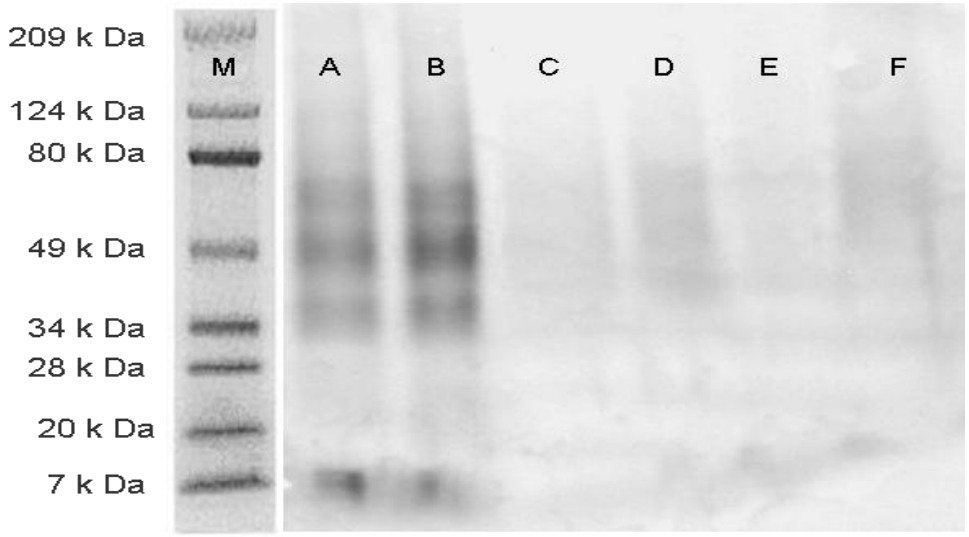

Fig. 5. SDS PAGE analysis of activated secondary paper sludge

M. Marker
A. Liquid Nitrogen+ detergent
B. Liquid Nitrogen+ detergent+protease inhibitor cocktail
C. Detergent
D. Detergent+ protease inhibitor cocktail
E. 7M urea+detergent
F. 7M urea+detergent+ protease inhibitor cocktail

FTIR Analysis

The FTIR spectra of three main process streams of the protein recovery tests are shown in Fig. 6. The residual stream (RS) represents the semi-solid mass settled out after removing the solubilized protein solution.

FTIR analysis based on the identification of bands related to the functional groups present in activated sludge and extractable proteinaceous components is supported by previous work (Hong *et al.* 1995; Garnier *et al.* 2005; Edalatmanesh *et al.* 2010). The main characteristic absorption bands of recovered sludge protein are related to C=O stretching at 1656 cm^{-1} (primary amino group-amide I), angular deformation of N–H at 1545 cm^{-1}(secondary amines-amide II), and C–H deformation at 1455 cm^{-1}, which might be caused by the secondary amines of CH_2 groups in aliphatic chains.

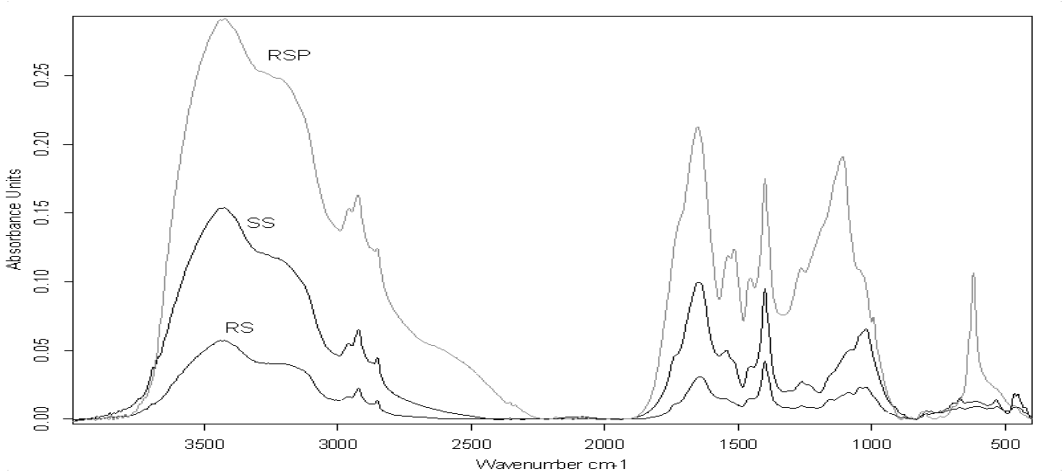

Fig. 6. FTIR spectra of recovered sludge protein (RSP), secondary sludge (SS), and residual mass (RS)

The band near 1405 cm^{-1} results from the N-C-H deformation in the protein. The band in the proximity of 1250 cm^{-1} originates from the asymmetric stretching vibration of C-O-C ester in the fat and C-O-C ether in the cellulose. The intense band near 1100 cm^{-1} is attributed to C-N stretching vibrations of both primary and secondary amines. Further, a sharp band near 670 cm^{-1} belongs to unsaturated C=C bonds.

The broad band, between 3600 and 3000 cm^{-1}, observed in all three materials, is attributed to free and bound O–H and N–H groups. SS is less intense in showing amide groups compared to RSP. Moreover the residual mass after protein recovery is devoid of any significant presence of amide II and C-O-C ester groups related to fats.

CONCLUSIONS

A high yield of protein recovery from paper SS was achieved through an extended hybrid method utilizing French pressing and sonication. An extensive optimization study revealed the process conditions to maximize protein solubilization and precipitation. This study confirms the technical feasibility to recover proteinaceous materials from paper mill sludge in appreciable quantities through a cost-effective method. Chemical and gravimetric analysis of the recovery process has confirmed that up to 90% of extractable protein can be recovered using commonly available reagents and equipment. A number of important findings through comprehensive characterization studies are reported below:

1. Paper mill SS contains considerable amounts of proteinaceous materials, which can be used as a resource for protein extraction.
2. Though lower in total protein content compared to municipal sewage sludge, recovered protein from paper sludge is qualitatively superior.
3. Recovered protein from paper sludge has significantly lower metal toxicity, especially in terms of heavy metals, compared to protein extracted from sewage sludge.

4. The protein recovery process used in this study also reduced the metal toxicity below detection limits, and in some cases the reduction was up to 4 to 6 times compared to raw sludge.
5. The presence of primary and secondary amide groups was confirmed through FTIR spectra, whereas amino acid analysis confirmed the presence of most essential amino acids in RSP in compatible concentrations to some protein products.
6. Both high and low MW proteins were also found in RSP of paper sludge.

The findings of this study are significant, as they highlight the potential for re-use and usefulness of abundantly available residual biomass from pulp and paper industries. Further studies to find the potential applications of recovered protein through an integrated biorefinery approach can ensure extra revenue for paper industry and at the same time mitigate environmental issues related to disposal of bio-solids.

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REFERENCES CITED

Abram, F., Gunnigle, E., and O'Flaherty, V. (2009). "Optimisation of protein extraction and 2-DE for metaproteomics of microbial communities from anaerobic wastewater treatment biofilms," *Electrophoresis* 30, 4149-4151

APHA (2005). *Standard Methods for the Examination of Water and Wastewater*, 21st Ed., American Public Health Association, Washington, DC.

Beauchamp, C. J., Charest, M. H., and Gosselin, A. (2002). "Examination of environmental quality of raw and composting de-inking paper sludge," *Chemosphere* 46, 887-895.

Benov, L., and Al-Ibraheem, J. (2002) "Disrupting *Escherichia coli*: A comparison of methods," *Journal of Biochemistry and Molecular Biology* 35(4), 428-431.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analyt. Biochem.* 72, 248-254.

Chishti, S. S., Hasnain, S. N., and Khan, M. A. (1992). "Studies on the recovery of sludge protein," *Water Res*. 26(2), 241-248.

Cheng, E. (2004) "Adhesion mechanism of soy protein adhesives with cellulosic materials," *PhD Thesis, Kansas State University* UMI # 3132160.

Christiansen, E. B., and Mitchell, D. H. (1978). "The production of protein from municipal sewage," *Am. Inst. Chem. Engrs Syrup. Ser*. 74, 175-181.

Deng, J., Geng, X., and Zhang, S. Y. (2007). "Characteristics of paper mill sludge and its utilization for the manufacture of medium density fiberboard," *J. Wood Fiber Sci.* 39(2), 345-351.

Edalatmanesh, M., Sain, M., and Liss, S. N. (2010). "Cellular biopolymers and molecular structure of a secondary pulp and paper mill sludge verified by spectroscopy and chemical extraction techniques," *Water Science & Technology* 62(12), 2846-2853.

Florkin, M., and Stoz, E. H. (1963). *Comprehensive Biochemistry, Proteins (Part 1)*, Elsevier, New York.

Garcia-Becerra, F. Y., Acosta, E. J., and Allen, D. G. (2010). "Alkaline extraction of wastewater activated sludge biosolids," *Bioresource Technology* 101, 6983-6991.

Garnier, C., Gorner, T., Lartiges, B. S., Abdelouhab, S., and Donato, P. (2005). "Characterization of activated sludge exopolymers from various origins: A combined size-exclusion chromatography and infrared microscopy study," *Water Research* 39, 3044-3054.

Geng, X., Deng, J., and Zhang, S. Y. (2007). "Paper mill sludge as a component of wood adhesive formulation," *Holzforschung* 61(6), 688-692.

Hong, S. G., Young, J. D., Chen, G. W., Chang, I. L., Hung, W. T., and Lee, D. J. (1995). "Freeze/thaw treatment on waste activated sludge: A FTIR spectroscopic study," *Journal of Environmental Science and Health. Part A: Environmental Science and Engineering and Toxicology* 30(8), 1717-1726.

Hwang, J., Zhang, L., Seo, S., Lee, Y., and Jahng, D. (2008). "Protein recovery from excess sludge for its use as animal feed," *Bioresource Technology* 99, 8949-8954.

Jung, J., Xing, X. H., and Matsumoto, K. (2001). "Kinetic analysis of disruption of excess activated sludge by Dyno Mill and characteristics of protein release for recovery of useful materials," *Biochem. Eng. J.* 8, 1-7.

Knorr, D., Kohler, G. O., and Betschart, A. A. (1977). "Potato protein concentrate: The influence of various methods of recovery upon yield compositional and functional characteristics," *J. Fd. Proc. Presern.* I, 235-247.

Lau, D. C. W. (1981). "Utilization of sewage sludge as a resource for protein extraction and recovery," *Conservation & Recycling* 4(3), 193-200.

Lerch, R. N. (1991). "Characterization of sewage sludge proteins," *Ph.D. diss*. Colorado State Univ., Fort Collins (Diss. Abstr. 9207333).

Lerch, R. N., Barbarick, K. A., Azari, P., Sommers, L. E., and Westfall, D. G.(1993a). "Sewage sludge proteins: I. Extraction methodology," *J. Environ. Qual*. 22, 620-624.

Lerch, R. N., Barbarick, K. A., Azari, P., Sommers, L. E., and Westfall, D. G.(1993b). "Sewage sludge proteins: II. Extract characterization," *J. Environ. Qual*. 22, 625-629.

Lo, Y. M., Cao, D., and Hahm, T. S. (2005). "Recovery of protein from poultry processing wastewater using membrane ultrafiltration," *Bioresource Technology* 96, 687-698.

Onyeche, T. I., Schlafer, O., Bormann, H., Schroder, C., and Sievers, M. (2002). "Ultrasonic cell disruption of stabilized sludge with subsequent anaerobic digestion," *Ultrasonic* 40, 31-35.

Potter, D. P., Wignall, J. A., and Windsor, M. L. (1974). "Wastewater in the fish industry," *Symp. Treatment of Wastes from Food and Drink Industry*, Univ, of Newcastle-upon-Tyne, U.K. (1974).

Rashid, M. T., Barry, D., and Goss, M. (2006). "Paper mill biosolids application to agricultural lands: Benefits and environmental concerns with special reference to situation in Canada," *Soil & Environ.* 25(2), 85-98.

Sastry, L. V. S., and Virupaksha, T. K. (1967). "Disc electrophoresis of sorghum seed proteins in polyacrylamide gel," *Analyt. Biochem.* 19, 505-513.

Shier, W. T., and Purwono, S. K. (1994). "Extraction of single-cell protein from activated sewage sludge: Thermal solubilization of protein," *Bioresour. Technol*. 49, 157-162.
Stafford, D. A., Wimpenry, W. T., and Hughes, D. E. (1979). "Protein recovery from biological treatment processes," *Conservation & Recycling*. 3, 33-43.
Stine, J. J., Pedersen, L., Smiley, S., and Bechtel, P. J. (2012). "Recovery and utilization of protein derived from Surimi wash-water," *Journal of Food Quality* 35, 43-50
Zhang, P., Zhang, G., and Wang, W. (2007). "Ultrasonic treatment of biological sludge: Floc disintegration, cell lysis and inactivation," *Bioresour. Technol*. 98, 207-210.
Zubay, G. (1983). "Biochemistry," pp. 143-162. The Benjamin/Cummings PubI. Co., Menlo Park, CA.

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