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Abstract—**The efficiency of excess sludge disintegration play a major role in protein extraction qualification. Hence, the use of optimal method is likely to promote the study of protein extractive rate in excess sludge. The objective of this study was to develop protein extraction by papain hydrolysis. There are four different factors were correlated to the protein yield during papain hydrolysis. These factors are protease content ,the ratio of solid to liquid, reaction time and temperature. The ratio of solid to liquid had a more significant influence on the total amount of protein than papain content. protein extraction concentration ranged from 8.23g/l to 9.98 g/l, and protein recovery rate was in the range of 42.3-52.7%.According to these data corresponded with high crude protein. In addition, the results showed that heavy metals of extractive protein was analysed below limited value. Thereby, papain hydrolysis treatment is recommended for practical extraction method because of its shorter extraction time. The nutrient composistion of protein recovered from excess sludge were feasible with animal protein feed.**

Keywords- papain; hydrolysis;protein exaction;excess sludge

I. INTRODUCTION

With the acceleration of urbanization and the treatment amount of wastewater,the significant increase in the account of sewage sludge production in wastewater treatment plants(WWTPS).The annual production of sewage sludge from WWTPS in china increased by approximately 125% from 2000 to 2007 and is expected to reach 10.5×10^4tons/day in 2012[1].The production of excess sludge has been estimated to account for about 1% of the volume of wastewater. Furthermore ,the treatment of excess sludge is reportedly as high as 25-60% of the total cost of wastewater treatment operation[2].Excess sludge, component rather complicated, contains heavy metals, pathogen and persistent organic pollutant etc[3].Therefore, improper disposal of the large amount of sludge generated a significant threat to air and water during wastewater treatment poses.

Excess sludge is comprised of abundant N、 P、 K elements and a wide variety organic substances such as proteins, polysaccharides ,humic acids and nucleic acids, etc[4].The protein content is known to account for between 20 and 60% of the weight of dry sludge[5].Protein is one of the most useful constituents in animal feed additive, meanwhile it is also high valuable resource. Recent years, the increasing world demand for food and feed protein has lead to the search for nonconventional protein sources to supplement the available protein sources. Much interest has been focused on the potential of converting agriculture, industrial or municipal wastewater sludge wastes to microbial protein. And it has concluded that the only essentially unutilized source of protein large enough to alleviate the shortage is cellular proteins in the mixed microbial cultures of activated sludge from WWTPS[6].

Protein have been reported to be a predominant organic component of the cell matrix, thereby solubilization of cellular materials (pretreatment before producing protein)in excess sludge must be carried out first, if you want to produce protein by use of excess sludge [7]. There are there are some researches about kinds of pretreatment methods which include physical, chemical, biological hydrolysis or combination of any two of these methods. Chishti et al.(1992) studied the Alkaline pretreatment of sludge at pH12.5 and the time of 24h.At the optium conditions, a maximum protein solubilization(90%) was obtained. Thomas et al.(1994) researched the thermal digestion of sewage sludge at 150-155℃ and ambient pressure for 20 min, and gained theoptimal extraction of high molecular weight protein. Chenyuhui et al.(2006) put forward circulation hydrolytic-acidification approach to pretreat excess sludge. Zhaoshunshun et al.(2008) extracted protein from residual sludge by alkali、acid and alkali combine ultrasonication hydrolysis. The maximal protein extractive ratio was 62.71%.El-Nawwi et al.(1996) examined cellulose recovery protein by alkali-treated bagasse under various culture conditions. The results indicated that an extraction protein content of 21-28%.

The protein-eatraction mechanism is that these treatment processes usually destroy the cell walls leading to the solubilization of extracellular and intracellular materials into the aqueous phase. Protein extraction rate of sludge was controlled the degree of cell disruptions[8].Hence, different extraction methods obviously influence the protein extractive rate. Early a few investigators such as Beszedits et. al(1981) and Ray et. al(1982) studied that utilization this protein source feeding sludge to animals. In fact, Shier et. al(1994) reported that the toxic metals or organic pollutants in the excess sludge are presumably concentreated in extraction protein[5].Hence, using of this protein source in the form of an animal feed supplement requires an economical and practical process to extract the protein in a form free of toxic materials without producing significant amount of side or residues products[9].

General remarks in literature indicate that protein of sludge are commonly extracted by chemical hydrolysis method, which was under the high temperature in response process. Therefore, the main objective of this study was to investigate the papain hydrolysis extracting protein of excess sludge under mild conditions. The method was used to rapidly release the intracellular proteins under mild conditions. This work will also give more information on the total amount of extractable proteins which can be released from excess sludge. In addition, the heavy metals concentration was investigated in a same extracted sludge sample.

II. MATERIAL AND METHODS

A. Sampling and Chemical Analysis of Excess Sludge

Sludge samples were collected from digested and dewatered sludge of one WWTP in Qingpu district, shanghai, P.R. China. The plant treat $2.5\times10^4 m^3/d$ of wastewater 94% from domestic and 6% from industrial origins using anaerobic-anoxic-oxic process. The collected samples were transported to the laboratory after sampling and stored at 4℃ until used.

pH value was measured by pH monitor (PHS- 3C, leici Co., Ltd., shanghai, china).Moisture was analyzed following the weight methods.Total solid (TS) were measured in an air-drying ovenat 105 ℃ and volatile matter(VM) were measured by calcinating at 600 ℃[10].Crude protein content of original sample was determined according to the standard protein assay by Kjeldahl determination. The protein is given Equation(1)[11]:

$$\mathrm{Pr}=\frac{cv\times0.014\times6{,}25}{m}\times100(\%) \quad (1)$$

Where Pr is the protein content of the sludge sample, C is the concentration of HCl i..e 0.1mol/l,V is the volume of HCl, M is the weight of the sludge sample.

The primary characteristics of excess sludge are described in Table I.

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TABLE I. CHARACTERISTICS OF EXCESS SLUDGE

PH	Moisture(%)	TS(g/l)	VM(g/l)	Sludge Protein[a](%)
6.84	83.95	189.76	142.32	43.61

a. sludge protein:in dry sludge weight

The dry sludge were obtained by natural venting method. Then dry sludge were subsequently sieved through a 2.0mm nylon wire mesh. The sludge were treated using oxidizing agent consisting of HNO_3,HCl and $HClO_4$ at the volume ratio of 15:5:4.Heavy metals concentration of sludge oxidized solution were deterimined by ICP-AES, Prodigy (Prodigy,Leeman Co., Ltd., USA).The tested result of heavy metals listed in Table II.

TABLE II. HEAVY METALS CONCENTRATION OF SLUDGE SAMPLE

Cu (mg/kg)	Zn (mg/kg)	Pb (mg/kg)	Cr (mg/kg)	Cd (mg/kg)	Hg (mg/kg)	As (mg/kg)	Ni (mg/kg)
1.924	1.129	≤0.001	0.504	0.001	0.001	0.001	0.093

B. *Papain hydrolysis process and production compent analysis*

Some principal extraction test factors were seclected for gaining optimal extraction rate in this study. The orthogonal experiments was designed, which was four factors and three levels. Nine time experiment and analyses were carried out in the duplicate. Hydrolysis reactions were performed at desired pH and temperature. Before hydrolysis, the original excess sludge were mixed with 40,60and 80ml distilled water and 0.4,0.5 and 0.6 g papain, respectively. The mixture was directly poured into a 100ml closed Erlenmeyer flask. The Erlenmeyer flask were then placed in a constant temperature water bath oscillator for controlling reaction temperature at 50, 55 and 60℃respectively Simultaneity,the papain hydrolysis experiments were continous achieved for 3.5,4.5 and 5.5h.Next,disinfection protease at boiling water reactor 10min.Finally,protease hydrolysis productions were centrifuged at 3600 rpm for 15 min. After centrifuged, the protein content of supernatant were measured by the modified lowry method. According to the objective of this research note essentially the protein extraction rate and concentration are discussed as reference index. The protein extraction rate(W_1) and the protein concentration(W_2) were expressed Equation(2) and Equation(3), respectively. The heavy metals concentration from supernatant were determined by above in duplicate method (mentioned in A) [12].

$$W_1 = \frac{W_l}{W_s} \times 100(\%) \quad (2)$$

$$W_2(g/l) = \frac{W_p}{V_l} \quad (3)$$

Where W_l is the hydrolysis centrifugal liquid(supernatant) weight, W_s is the protein content of dry sludge. Where W_p is the protein content of supernatant, V_l is the volume of supernatant.

C. *Isoelectric point determination of Protein*

Isoelectric point of Protein(shoerted as IPP) was determined by adjust pH method.in order to determine IPP of the solute protein in the supernatant under the optimum hydrolysis conditions,50ml of supernatant was divided into fifths. The pH of soluble protein liquid was adjusted to 4.5,5.0 5.5 ,6.0and 6.5 by 0.1mol/l HCl or 0.1mol/l H_2SO_4. After stirring 10000 rpm for 15 min at 4℃,the mixture was centrifugalized and one new supernatant can be gained.The new supernatant protein concentration was determinated according to the modified method. It was estimated that the ratio of protein precipitate at different PH values. Therefore, protein isoelectric point was judged at last according to the relation between the pH and The protein precipitation rate. The protein precipitation rate(P_t) was expressed as follows: [12].

$$P_t = \frac{P_{r0} - P_{ri}}{P_{ro}} \times 100(\%)$$

Where P_{ro} and P_{ri} are the protein concentration before and after the centrifugation, respectively.

After precipitation, the high-water content protein can be gained.The protein precipitate was intituled as hemi-solid, which was then tested moisture following the china animal feed standard[13].The protein precipitate was dried for 48 hours at 50℃ in this study.

III. RESULTS AND DISCUSSION

A. *The excess sludge component analysis*

The excess sludge was collected from the waste actived sludge of WWTP, shanghai. Table 1 shows general background information for excess sludge sampled for this study. Different sludge samples have difference characteristics. From Table 1, pH of the sludge is 6.84 and approximately neutrality. Its water content is 83.96%. Table 2 shows the heavy metals content t the content of heavy metals is not high[15].

B. *Optimum hydrolysis process conditions*

Table 3 presents the protein extraction rate in orthogonal experiments. The results showed that protein recovery rate is in the range of 30.9% to 52.73%.The results showed that the papain hydrolysis content and the ratio of solid to liquid have obviously influence on the protein extraction rate. The optimal protease hydrolysis process conditions for extraction of sludge protein were as follows: 55 ℃ of temperature, 5.5h of reaction time, 6% of papain content and 1:4 of the ratio of solid to liquid. Under these conditions, the maximal protein extraction ratio was 52.73%.This extraction value is similar to that gained by Zhao shunshun et al.(2008). After papain hydrolysis, the protein concentration are summarized in Figure 1 as papain content increased,which is in the range of 5.484 -7.029g/l. The protein concentration are summarized in Figure 2 as the ratio of solid to liquid increased, which is in the range of 3.967 -8.867g/l. According to R value, we can estimate the ratio of solid to liquid is prominent affect protein extraction quantification than papain content.

TABLE III. RESULT OF THE PAPAIN HYDROLYSIS ORTHOGONAL EXPERIMENTS

Test number	Factors levels				result
	Papain content(%)	*Temperatuer (℃)*	*reaction time(h)*	*the ratio of solid to liquid(W/V)*	*protein extraction ratio(%)*
1	4	50	3.5	1:4	42.3
2	4	55	4.5	1:6	35.7
3	4	60	5.5	1:8	30.9
4	5	55	3.5	1:8	28.25
5	5	60	4.5	1:4	45
6	5	50	5.5	1:6	51.21
7	6	60	3.5	1:6	51.71
8	6	50	4.5	1:8	38.43
9	6	55	5.5	1:4	52.73

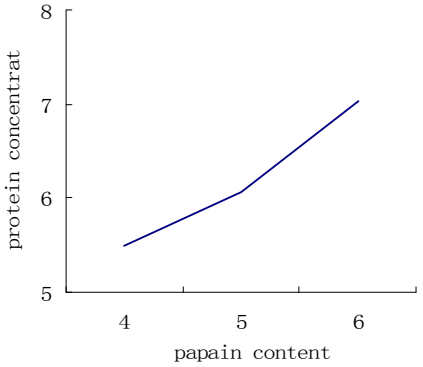

Figure 1. Effect curve of the papain content

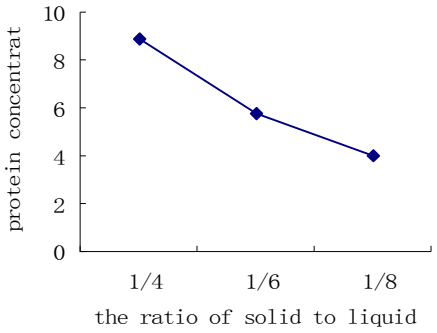

Figure 2. Effect curve of the ratio of solid to liquid

C. *Extraction of protein sediment*

The protein sediment were separated by adjust the pH of supernatan. IPP of the solute protein in the supernatant was critical role in protein extraction,while the pH did play an important role in IPP of the solute protein. It was observed that the solute protein were maximal separated at the optimal pH condition. The Figure 3 presents the protein concentration of supernatant in the different PH values. Meanwhile, the deposit of protein is maximum at pH 5.5. Amount of protein were deposited at protein isoelectric point. The protein isoelectric point was 5.5 in this study. By calculated, the protein precipitation rate is 84.96% at the protein isoelectric point.The protein deposit solid moisture is accord with feed protein standard. [16].

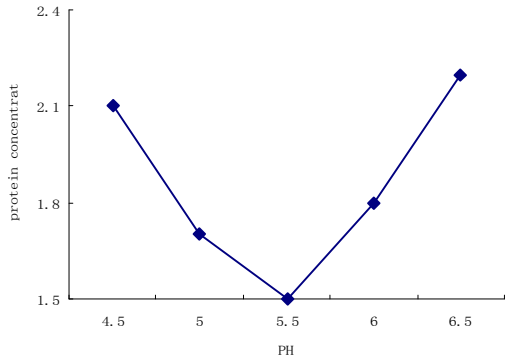

Figure 3. The change of protein concentration in supernate with PH values

D. *Extraction liquid and protein deposit characters*

The heavy metals concentration of centrifugal liquid were determined.The table IV summarized the experiment results of heavy metals concentration. There were almost not detection Cu、Pb、Cr、Cd、Hg and As in the papain extraction liquid. The concentration of other heavy metals, such as Ni 、Zn，were lower.In this study, there will give two example (experiment 3 and 9) to illustrate. Moreover, The hydrolysis experiments 3 has minor protein extraction ratio and the hydrolysis experiments 9 has maximal protein extraction ratio. The result of heavy metals concentration are given in Table IV,respectively. The Ni and Zn of concentration in experiment 3 are 0.06 and 0.01μg/l, meanwhile experiment 9 are 0.19 , 0.02μg/l.

TABLE IV. HEAVY METALS CONCENTRATION OF THE EXTRACTION LIQUID

Experiment number	Cu,Pb,Cr,Cd,Hg,As (μg/l)	Ni (μg/l)	Zn (μg/l)
3	≤0.01	0.06	0.01
9	≤0.01	0.19	0.02

From The Table IV,it is concluded that the heavy metals concentration, in the liquid phase, is also enhance as the protein extraction rate of excess sludge raise. The reason for the heavy metals is concentrated in excess sludge. During the protease hydrolysis, the heavy metals was easy transfered into liquid phase owing to sufficiently hydrolysis. Wangzhijun et al.(2004) indicated that the decrease of sludge particle during the hydrolysis and the release of intracellular high molecule matters processes could mainly attribute to the optimal protease hydrolysis process conditions. Furthermore, the sludge are broken first and then high molecule organic matters and slight heavy metals are released into the liquid phase during the papain hydrolysis.

IV. CONCLUSIONS

The results showed that the papain hydrolysis content and the ratio of solid to liquid have obviously influence on the protein extraction rate. Furthermore, the optimal protease hydrolysis process conditions for extraction of sludge protein were as follows: temperature 55 ℃, reaction time 5.5h, papain content 6% and the ratio of solid to liquid 1:4.The protein test is a temperance hydrolysis procedure which can be done at lower temperature and shorter time. The heavy metals concentration is accord with feed protein standard. Accordingly, it could a very effective hydrolysis protocols for excess sludge protein extraction .

ACKNOWLEDGMENT

The authors are thankful to the Innovation Foundation of Donghua University for PhD candidates, the Major Project of Educational Ministry (No. 107124), Project of Key Discipline Construction in Shanghai (No. B604) and National High Technology Research and Development Program of China (863 Program, No. 2006AA06Z132) for financial support of this study.

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