Iranica Journal of Energy & Environment

Journal Homepage: www.ijee.net

IJEE an official peer review journal of Babol Noshirvani University of Technology, ISSN:2079-2115

Crude and Pure Bioflocculants Produced from *Bacillus subtillis* for Low Concentration of Copper (Cu^{2+}) Removal

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P A P E R I N F O

Paper history:
Received 30 November 2014
Accepted in revised form 25 Janurary 2015

Keywords:
Copper
Bacillus subtillis
Crude bio flocculants
Pure bio flocculants

A B S T R A C T

Heavy metals can be found abundantly in earth and being utilized as sources for human being usage. One of the most frequently utilized metals use was copper. Copper was used as a conductor for electrical and electronically product, battery productions, utensils and ornamental purposes due to their conductivity and malleability. Even though copper was very important for human being, excessive exposure of copper to the environment would lead to environmental problems. Since copper possessed an ability to be accumulated into the environment and enter the food chain, efficient techniques to remove copper from wastewater are vital. This paper was focused on biological approaches to remove copper from wastewater using bio flocculants produced by *Bacillus subtillis*. Important factor such as pH, concentrations of inorganic salt, bio flocculants dosage and initial concentrations of copper were also being studied since copper removal was very dependable on stated variables. Best pH operated for pure bio flocculants was recorded at pH of 6, with 2 mL $CaCl_2$ and 5 g/L dosage of pure bio flocculants for 2, 3, 4 and 5 mg/L initial concentrations of copper. While the best pH operated for crude bio flocculants was recorded at pH 4, with 2mL $CaCl_2$ and 2 and 5 mg/L initial concentrations of copper.

***doi**: 10.5829/idosi.ijee.2015.06.02.05*

INTRODUCTION

Heavy metal can be introduced into the environment through natural occurring process or anthropogenic process. Heavy metals were believed to be an essential element for living organism since it presence would give benefit. In example, the presence of iron (Fe^{3+}) associated with haemoglobin protein in blood are functioned to transport oxygen to muscle and organ in human, presence of chromium (Cr) would facilitate the glucose uptake and presence of zinc (Zn) associated with DNA polymerase enzyme would facilitated the process of synthesizing DNA.

Copper known as the first metal to be mined and used to produced tools, weapons and ornamental items 1000 years ago due to it electrical conductivity, thermal conductivity, and corrosion resistance properties. Copper (Cu^{2+}) are commonly metal that been widely spread in the earth due to its availability as an essential element for plants and animal. Copper can be found in rock, water, and soil naturally. According to literature [1] copper known as transitions metals which can be divided into three transitions states which are cu^0 (metal), Cu^+ (cuprous ion) and Cu^{2+} (cupric ions). From these three transitions states, cupric ions know as the most hazardous species for the living organism. Even though copper can be hazardous to living organism, an appropriate concentrations of copper are still needed by organism due to its impotency as functional component to several essential enzymes which also known as copper enzymes.

Copper can be found abundantly through the environment due to its availability from natural sources or anthropogenic sources. Eruptions of volcanic mountain, degradations or decaying of plant and vegetations, forest fire and sea spray phenomenon could be a major factor for copper occurrences into the environment [2]. Mining industries, agricultural activities, electrical product productions, electro plating industries, wood industries, power plant stations and

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Please cite this article as: M. A. Azmi, I. Norli, Z. A. Farehah, S. A. Ishak, M. N. Siti Norfariha, A. T. Azieda, 2015. Crude and Pure Bioflocculants Produced from *Bacillus subtillis* for Low Concentration of Copper (Cu^{2+}) Removal, Iranica Journal of Energy and Environment 6 (2): 103-110.

incinerators were some example of anthropogenic sources of copper enter to the environment [3-6]. Due to its abundance, copper could be deposited and accumulated into water and soil which later entering the food chain [2, 6]. Besides, researchers [7, 8] have reported that, copper could also enter to food chain through the product which have been canned or process by copper containing container. As we know, when copper are introduce into the food chain, it could be very dangerous to organism due to its toxicity. Several research papers have reported that low concentrations copper poisoning could lead to skin itching and dermatization [1, 9]. Meanwhile high dosage of copper poisoning could lead to more serious internal organ malfunctioned and damage such as changes in kidney functions and lever damage [1, 10-12].

Nowadays, more alternative methods were developed in order to remove copper from aqueous solutions such as precipitations technique [13] phytoextractions, membrane filtrations, ultra filtrations, electro dialysis, reverse osmosis, adsorptions method, coagulations/filtrations method, ionic exchange [14], and solvent extractions [15]. Among these methods, adsorption technique known as the most adopted method use method to remove copper [16]. Since considering the statement stated in literature [17-19] inability of current conventional technique such as ion exchange, precipitations process, electrochemical process are not effective treating low concentrations of heavy metal (<100 mg/L) the use of microorganism to remove heavy metal form waste water seems more promising.

The search for an effective method to remove copper from wastewater is vitals due to its capability to accumulate into the environment. Copper removal from environment has been fall into a limelight due to it toxicity and ability to accumulate. This process becomes more challenging conjunct to several limitations of current method which are not effective when low concentrations of copper involved. Besides, the applications of conventional flocculating agent would resulted in some health and environmental problems such as Alzheimer disease [20], cancer, and the derivations of secondary pollutant [21]. In this research pH, initial metal concentrations, bio-flocculants concentrations, and inorganic salt concentrations were considered because of it factor essentiality [22]. Thus, the results from this study could provide a scientific basis as an alternative way to remove copper from aqueous solutions.

METHOD

Media and cultivations conditions

Bacteria strains (*Bacillus subtillis*) were cultured in 250 mL medium in 500 mL flask on rotary shaker (145 rpm) at 40°C for inoculations preparations. After 48 hours cultivations 4 % of inoculums was introduced into 100 mL of medium in 250 mL flask and incubated on rotary shaker (145 rpm) at 40°C for another 48 hours. The medium contained 10 g/L glucose, 0.25 g/L yeast extract, 25 g/L L-glutamic acid and 0.25 g/L magnesium sulphate heptahydrate ($MgSO_4.7H_2O$).During incubations process, the optical density of the cultured bacteria was measured using spectrophotometer at 600nm wavelength for every 12 hours time interval to ensure bacteria growth.

Metal solutions preparations

The stock solutions of Copper (Cu^{2+}) with 1000 part per million (ppm) concentrations were prepared by using copper (II) sulphate ($CuSO_4$) and deionized water. Later, the copper solutions were diluted into desired concentrations varied from 1 ppm to 5 ppm. Copper solutions pH was adjusted using 0.1 N NaOH (Sodium Hydroxide) and 0.1 N HCl (Hydrochloric acids). Later the concentration of copper ions was determined by using atomic absorbing spectrophotometer (AAS).

Extraction of the bio flocculants (Crude)

The culture broth of bacteria was centrifuged at 10000 rpm for 15 minutes at 5°C to separate bacteria cell and bio flocculants. After separations process two clear layer was appeared and supernatant layer was taken to extract crude bio flocculants. Extractions of crude bio flocculants from supernatant were involved precipitations process by using 4 volume of cold (-40°C) ethanol (95%). The mixture was later left for 24 hours before it was centrifuge again at 10000 rpm for 15 minutes (5°C). The supernatant later was diluted with 4 volume of cold ethanol and stored at 4°C overnight. Finally, the precipitations were taken for lyphylozations process.

Extraction of the bio flocculants (Pure)

The culture broth was centrifuged at 10000 rpm for 15 minutes at 5˚C in order to separate bacterial cell and bio flocculants. The supernatant layer were taken and dialyzed with cold (-40°C) ethanol (95%) overnight. The mixture later was centrifuges again at 10000 rpm for 15 minutes at 5°C. Shortly after that the supernatant was diluted with 4 volume of cold ethanol and stored inside the refrigerator for 24 hours. After spending 24 hours resettling inside the refrigerator two layers of solutions were appeared. The precipitation formed was taken and redissolved with 10% concentrations of cetylpyredinium chloride ($C_{12}H_{38}CINH_2O$) while stirred. After several hours, the precipitations of CPC complex were collected centrifugal process and dissolved with 0.5 M of NaCl (sodium chloride). Four volume of cold ethanol (-40°C) were then added in order to obtained precipitate. The precipitates later were undergoes lyophilisation process.

Determinations of pH for copper (Cu^{2+}) removal
Determinations of the best pH were involved various value of pH ranging from 4 to 9. One ppm of copper (Cu^{2+}) were prepared in a total volume of 1 litre synthetic wastewater. After that 1 mg of bio flocculants were added into the mix solutions with the addition of 4 mL concentrations of $CaCl_2$ before various pH were adjusted to 4, 5,6,7,8 and 9. Next, the solutions were agitated rapidly using jar test procedure at 200 rpm for 1 minute, agitated moderately at 90 rpm for another 5 minutes before it were left unstirred for 5 minutes. Copper removal value was analyzed using Atomic Absorption Spectrophotometer.

Determinations of dosage for copper (Cu^{2+}) removal
One ppm of copper (Cu^{2+}) were prepared in a total volume of 1 litre synthetic wastewater. After that various dosage of bio flocculants ranging from 1 to 5 mg were added into the solutions. Next, 4mL of $CaCl_2$were added and the pH was adjusted to the best pH obtained. Then the mix solutions were agitated rapidly using jar test procedure at 200 rpm for 1 minute and shortly after that, the agitated speed was reduced to 90 rpm for another 5 minutes. Later, mix solutions were left unstirred for 5 minutes before the copper removal value was analyzed.

Determinations of best $CaCl_2$ concentrations for copper (Cu^{2+}) removal
To start with, 1 ppm of copper (Cu^{2+}) were prepared in a total volume of 1 litre synthetic wastewater. Shortly after that best dosage of bio flocculants obtained previous experiment were added. Then, 1 to 5mL concentrations of $CaCl_2$were added into the mix solutions and the pH were adjusted to the best pH conditions as obtained. The mix solutions were agitated rapidly using jar test procedure at 200 rpm for 1 minute, agitated moderately at 90 rpm for another 5 minutes before it were left unstirred for 5 minutes. Finally the copper removal values were analyzed.

Determinations of best initial copper (Cu^{2+}) concentrations for copper (Cu^{2+}) removal
For this experiment, various concentrations of copper valued from 1, 2, 3, 4 and 5 ppm were prepared in a total volume of 1 litre synthetic wastewater. Then, the best concentrations of bio flocculants dosage and the best concentrations of $CaCl_2$ were added into the mix solutions. The pH of the solutions was later adjusted base on the result obtained. The solutions were agitated rapidly using jar test procedure at 200 rpm for 1 minute and agitated moderately at 90 rpm for another 5 minutes before it were left unstirred for 5 minutes before the copper removal value was analyzed.

Statistical analysis
Analysis of variance (ANOVA) test was carried out using SPSS version 16 (IBM, USA). The data considered as significantly difference as the P-value obtained are less than confidence level of 95% or 0.05. The analysis of interactions was also been carried out by using SPSS software.

RESULT AND DISCUSSION

Effect of pH towards bio flocculants copper removal capability
As shown in Figure 1, different type of bio flocculants would react contrarily to vary range of pH. Crude bio flocculants seems recorded a reduction on copper removal percentage while pure bio flocculants seems recorded an increase in copper removal percentage as the pH value increase. At the beginning (pH 4), both type of bio flocculants recorded almost the same value of copper removal with 67 and 68% removal. After word, the removal percentage of crude bio flocculants was later dropped to 56% at pH 6 and 43 % at pH 8. At the end, at pH 10 there are no removal percentage was recorded. On the other hands, the removal percentage of pure bio flocculants was recorded increase at pH 6 with 74% removal percentage and until there are no noticeable changes occur. But at pH 10 the removal percentage was drastically reduce to 0% removal same as same as crude bio flocculants case. Both types of bio flocculants recorded highest removal percentage at pH 4 and lowest removal percentage at pH 10. According to statistical analysis on Table 1, the main effect of pH would play a major factor in copper removal efficiency as the P-value recorded less that 0.05 confidence interval. This indicates that, any changes in pH value would give a significant effect towards copper removal capabilities. There are also strong interactions between pH and type of bio flocculants use since the P-value for pH * BF type is less than 0.05 which demonstrate that changes in pH might influence the type of bio flocculants use to perform. Factor such as pH seems to be a major influence for metal removal due to ability of active site to remove or receive protons as reported in literature [23, 24]. Reduction of copper removal percentage on crude bio flocculants might be due to several factors such as through alkaline degradations process and the congestions process of hydroxides ion to the active binding sites. This phenomenon was explained by several researchers which find that in alkaline condition, the chain of polysaccharide that formed polymer would be hydrolyzed and break into monomer component. In these cases, the glycosidic

linkage between monomer would be break and the bio flocculants polymer would be degraded and reduce their ability to remove copper ions [25]. Besides, the oversaturation process of hydroxides ions to the active sites as stated in literature [26]might also occur. During the alkaline conditions, the amount of hydroxides ion would be higher compared to natural conditions. At this point, the binding sites of the bio flocculants might be occupied by the excessive amount of hydroxides ions which resulting lower removal capabilities of copper ions. This is very different from pure bio flocculants which is more tolerate able to pH changes. As can see above, at pH 6, 7, 8 and 9 the removal percentage doesn't seems give a much different and it might be due to the properties of pure bio flocculants itself which have more binding sites available compare to the crude ones resulting more stabilize removal activities during increasing of pH. The interactions plot as illustrated in Figure 2 suggest that, copper removal percentage for crude bio flocculants tend to decline as the pH increase but that of the pure bio flocculants remains pretty much the same.

Table 1 . ANOVA Table on tests of between-subjects effects toward pH and types of bio flocculants.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	24627.738[a]	13	1894.441	164.055	.000
Intercept	121501.929	1	121501.929	1.052E4	.000
pH	20778.905	6	3463.151	299.902	.000
BF type	2273.357	1	2273.357	196.868	.000
pH * BF type	1575.476	6	262.579	22.739	.000
Error	323.333	28	11.548		
Total	146453.000	42			
Corrected Total	24951.071	41			

a. R Squared = 0.987 (Adjusted R Squared =0 .981)

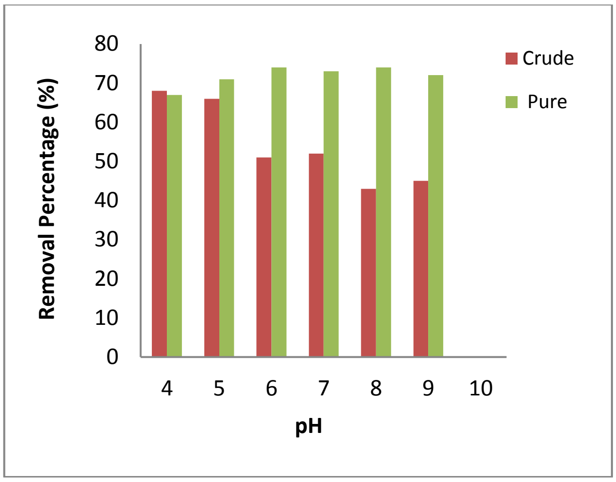

Figure 1. Effect on pH toward copper removal efficiency for crude bio flocculants and pure bio flocculants.

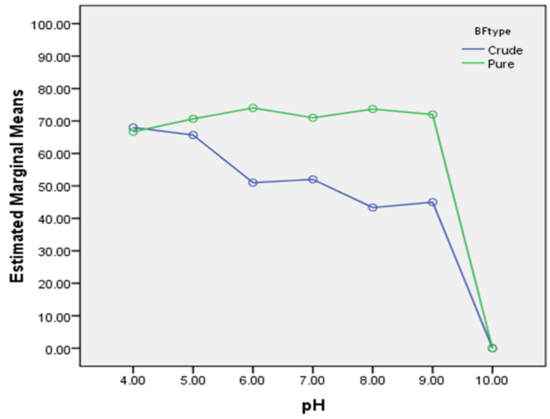

Figure 2. Interactions plot between pH and types of bio flocculants.

Effect of dosage towards bio flocculants copper removal capability

Figure 3 depicts two types of bio flocculants were test in order to determine their dosage efficiency toward copper removal efficiency. Each bio flocculants type would give different result on removal percentage. For crude bio flocculants, at the beginning of dosage of 1 mL, the removal percentage was recorded at 56% and this value was later increased to 64% as the dosage increased until it was barely changed (3 and 4 mL). But, at the end, the percentage removal was sharply depleted to 57% at 5mL dosage of bio flocculants. Other while, pure bio flocculants shows a different trend as its start at 79% removal at 1 mL dosage and recorded a slightly changes at 2mL dosage. Shortly after that, the removal percentage was decline to 71% at 3mL dosage of bio flocculants. At the end, the removal percentage was raise to 74 and 77% for 4 and 5 mL dosage of bio flocculants. According to statistical analysis given in Table 2, the main effect of bio flocculants dosage would play a major role in copper removal efficiency since the P-value was recorded less than 0.05 confidence intervals. These demonstrate that any changes in bio flocculants dosage might give a significant effect towards copper removal capabilities. The interactions between type of bio flocculants and dosage of bio flocculants use were also been studied. From the same table, it is demonstrated that the interactions between these two factor was possessed a strong relationships since the P-value for BF dosage * BF type was less than 0.05 confidence intervals which exhibit any changes in dosage of bio flocculants would affect the type of bio flocculants to perform. The fact is, an increase in bio flocculants dosage would provide much amount of binding sites which later would increase the removal percentage. However, surplus amount of bio flocculants provide resulting on the excessive amount of active sites compared to metal ions available. This occurrence was explained by previous studies reported in literature [27, 28]; which stated that any reduction in removal percentage with the increasing of cell concentrations may be due to insufficient metal ion in solutions compared to the availability of the binding sites. The removal percentage of crude bio flocculants start to rise as we introduce more dosage of bio flocculants, and this value start to depleted as the dosage increase. At this stage excessive supply of crude bio flocculants dosage would provide an excessive amount of binding sites and this disrupt chemical equilibrium which subsequently reduces the removal percentage and this statement was supported by the study carried out from [29-31]. The interactions plot as illustrated in Figure 4 imply that, copper removal percentage for crude bio flocculants start to rise as the dosage increased, but after that it would depleted until the end.

Table 2 . ANOVA Table on tests of between-subjects effects toward bio flocculants dose and types.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2196.667[a]	9	244.074	43.585	.000
Intercept	141453.333	1	141453.333	2.526E 4	.000
BF dosage	89.667	4	22.417	4.003	.015
BF type	1856.533	1	1856.533	331.52 4	.000
BF dosage * BF type	250.467	4	62.617	11.182	.000
Error	112.000	20	5.600		
Total	143762.000	30			
Corrected Total	2308.667	29			

a. R Squared = 0.951 (Adjusted R Squared =0.930)

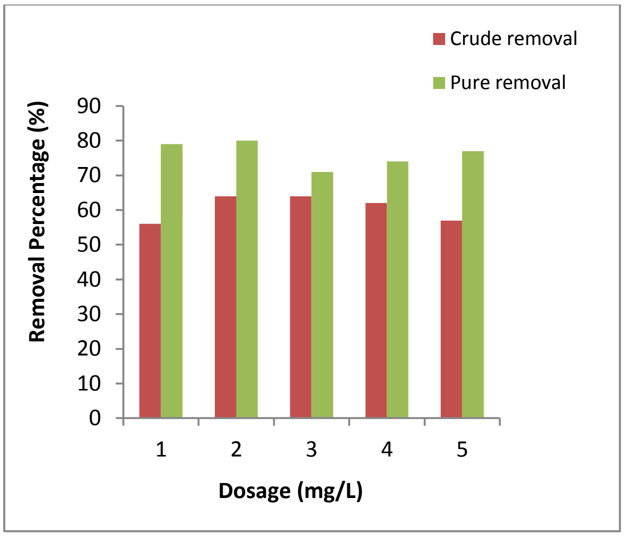

Figure 3: Effect on crude bio flocculants dosage toward copper removal efficiency for crude and pure bio flocculants.

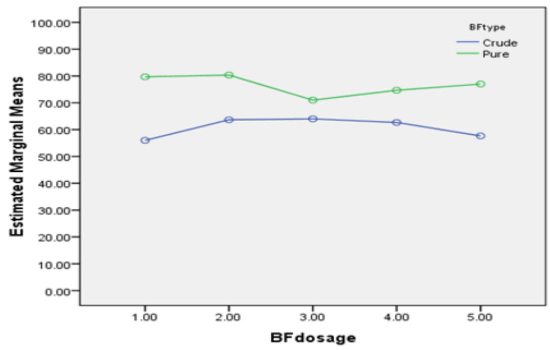

Figure 4. Interactions plot between bio flocculants dosage and types of bio flocculants

Effect of inorganic salt dosage towards bio flocculants copper removal capability

According to Figure 5, effects of inorganic salt additions towards copper removal percentage for different types of bio flocculants use were recorded. Each type of bio flocculants were reacts differently to inorganic salt additions and in this case, pure bio flocculants recorded much higher removal percentage of removal compared to crude bio flocculants. At the beginning both bio flocculants recorded almost the same percentage of removal at 63 and 66%. As the dosage of $CaCl_2$ increased drastically to 83% removal, but after 3 mL dosage of $CaCl_2$ introduce, the removal percentage was declined to 68%. Finally, the removal percentage remains unchanged at 4 and 5 mL dosage of $CaCl_2$ with 81% removal percentage. Different trend of removal percentage was recorded for crude bio flocculants. As the dosage of $CaCl_2$ increased, the removal percentage recorded unnoticeable changes with the range of percentage removal from 61 to 63%. Statistical analysis result from Figure 5 shows that the main effect of $CaCl_2$ dosage plays a role towards copper removal percentage since the P-value recorded less than 0.05 confidence intervals. This indicates that changes in $CaCl_2$ dosage will also give a significant effect toward copper removal capabilities. The interactions effect between $CaCl_2$ dosage and types of bio flocculants use were also revealed that they possessed a strong interactions since the P-value for $CaCl_2$dosage * BF type was less than 0.05 confidence intervals. Presence of inorganic salt during the flocculation process act as a stabilizer to neutralized the charges hence aid in binding process [32, 33]. According to literature [34], they found that inorganic salt such as $CaCl_2$ help metal ion removal by naturalizing and stabilizing the charges for binding process at active sites. This explained why there was a fluctuation in removal percentage for pure bio flocculants.

Several researchers have reported that presence of inorganic salt may induce the removal percentages [35-38]. Usually inorganic salt possessed a negative charges ion and these charges are completely different from metal ion which possessed a positive charges ion. When the reaction begins, the positive ions will bind with the negative ions and produce a natural charges complex and this would facilitate
the flocculation process resulting increase in removal percentage. The interactions plot illustrated in Figure 6 suggested that as the dosage of $CaCl_2$ increase the removal percentage of crude bio flocculants pretty much the same while pure bio flocculants are drastically changed.

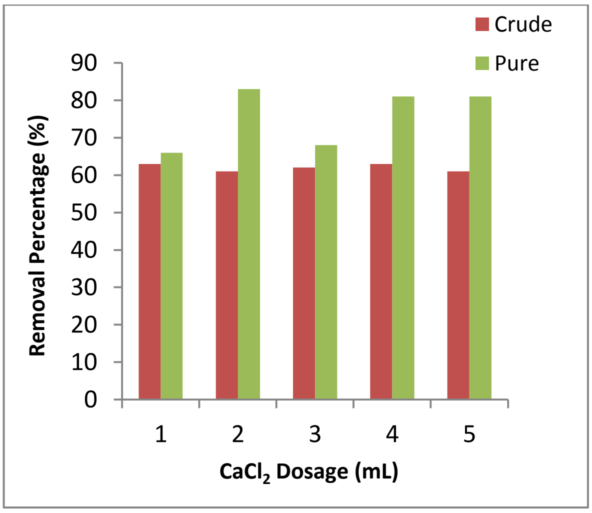

Figure 5. Effect on inorganic salt ($CaCl_2$) dosage toward copper removal efficiency for crude bio flocculants and pure bio flocculants.

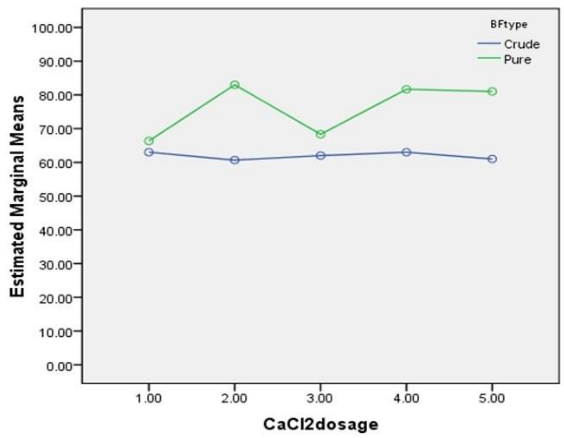

Figure 6. Interactions plot between inorganic salt dosage and types of bio flocculants.

TABLE 3. ANOVA Table on tests of between-subjects effects toward $CaCl_2$ and types of bio flocculants.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2287.333[a]	9	254.148	401.287	.000
Intercept	142830.000	1	142830.000	2.255E5	.000
$CaCl_2$ dosage	339.667	4	84.917	134.079	.000
BF type	1498.133	1	1498.133	2.365E3	.000
$CaCl_2$dosage * BF type	449.533	4	112.383	177.447	.000
Error	12.667	20	.633		
Total	145130.000	30			
Corrected Total	2300.000	29			

a. R Squared = 0.994 (Adjusted R Squared = 0.992)

Effect of initial copper concentrations towards bio flocculants copper removal capability

Based on Figure 7 above, the effect of initial copper concentrations toward copper removal efficiency was recorded. Different types of bio flocculants use might gift different reactions. Pure bio flocculants show a more stable removal percentage compare to crude bio flocculants. At the beginning, pure bio flocculants recorded 98% removal percentage at 1mg/L initial copper concentrations. Then, at 2, 3, 4 and 5 mg/L initial copper concentrations, the removal percentage remain stagnant (99% removal). This is obviously different compare to crude bio flocculants which recorded 91 % removal percentage at 1 mg/L initial copper concentrations. Then, the removal percentage was increased to 99% removal at 2 mg/L initial copper concentrations but start to decline to 97% removal when the initial copper concentration increased to 3 and 4 mg/L. Finally, the removal percentage increased again to 99 % removal percentage at 5 mg/L initial copper concentrations. Based on statistical analysis above, the main effect of initial copper concentrations give a significant effect toward copper removal percentage since the p value is much smaller than 0.05 confidence intervals. This indicates that changes in initial copper concentrations would lead to significant changes in copper removal efficiency. A study carried out by pervious researcher found that increase in metal ions concentrations resulted in increase removal efficiency until the binding sites become saturated followed by reductions of removal percentage [26, 35]. This was the explanations for 2mg/L initial concentrations of copper crude bio flocculants removal percentage was depleted. At this stage, the binding sites become fully occupied by the copper ions and the excessive copper ions were unattached which make it remains inside the aqueous mixture solutions resulting in low removal efficiency. Pure bio flocculants seem to have a more stable removal percentage compare to crude bio flocculants. It was suggested [39] pure bio flocculants have low moisture content and due to this properties [40]explained that loss of moisture may lead to greater affinity, plot as illustrated from Figure 8 illustrated that as the initial concentrations of copper ions increase the removal percentage of crude bio flocculants but later depleted due to oversaturation, but it keep remains stagnant for pure bio flocculants type.

TABLE 4. ANOVA Table on tests of between-subjects effects toward initial copper dose and types of bio flocculants.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	151.867[a]	9	16.874	168.741	.000
Intercept	286554.133	1	286554.133	2.866E6	.000
Cu dosage	74.867	4	18.717	187.167	.000
BF type	34.133	1	34.133	341.333	.000
Cu dosage * BF type	42.867	4	10.717	107.167	.000
Error	2.000	20	.100		
Total	286708.000	30			
Corrected Total	153.867	29			

a. R Squared = 0.987 (Adjusted R Squared =0 .981)

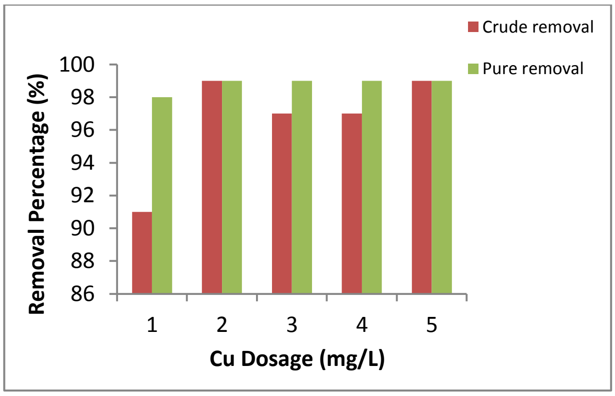

Figure 7. Effect on initial copper concentrations toward copper removal efficiency for crude bio flocculants and pure bio flocculants.

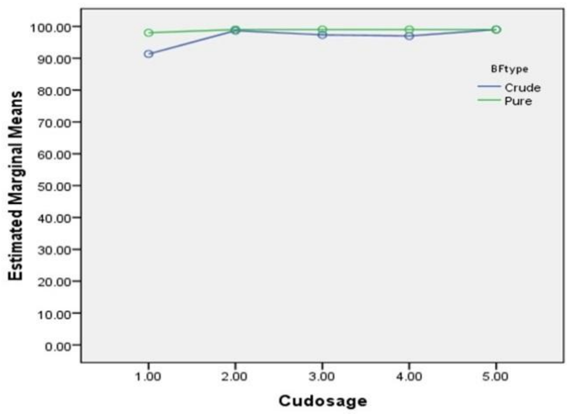

Figure 8: Interactions plot between copper dosage and types of bio flocculants.

CONCLUSION

Result from this study provide an information regarding the factor that affecting metal removal process by using crude and pure bio flocculants extract from *Bacillus subtillis.* Factor such as pH, concentrations of inorganic salt, dosage of bio flocculants and initial concentrations of copper ion would give a significant effect toward copper removal capabilities. Crude and pure bio flocculants extract from *Bacillus subtillis* bacterium may find possible applications as an alternative ways to remove copper at lower concentrations but further study need to be carried out on its actions mechanism, scaling up process and modifications to enhance its ability in order to make it more reliable into the industrial utilization.

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Persian Abstract

DOI: 10.5829/idosi.ijee.2015.06.02.05

چکیده

فلزات سنگین به طور گسترده ای در زمین وجود دارد و به عنوان منابعی جهت استفاده ی بشر می باشند. یکی از پر مصرف ترین این فلزات مس است. مس به عنوان یک هادی الکتریکی در محصولات الکتریکی ، تولید باطری و وسایل تزیینی به دلیل هدایت پذیری و اختلاط پذیری به کار می رود. اگرچه مس برای انسان بسیار مهم است اما استفاده ی بیش از حدآن می تواند منجر به مشکلات زیست محیطی گردد. با توجه به اینکه مس توانایی تجمع در محیط زیست و ورود به چرخه ی غذایی را دارد، استفاده از تکنیک هایی جهت حذف آن از فاضلاب حیاتی است. این مطالعه بر مبنای رویکرد حذف بیولوژیکی مس از فاضلاب با استفاده از لخته های بیولوژیکی تولید شده توسط *Bacillus subtillis* می باشد. فاکتورهای مهم نظیرpH، غلظت، نمک های غیرآلی، مقدار لخته های بیولوژیکی و غلظت اولیه مس مورد بررسی قرار گرفت، چرا که حذف مس به متغیرهای ثابت بسیار وابسته است.بهترین pH اجرا شده برای لخته های بیولوژیکی خالص برابر با ۲میلی لیترکلسیم کلرید وg/L۵از مس می باشد. در حالی که بهترین pH انجام شده برای لخته های بیولوژیکی خام برابربا۴ با۲ میلی لیترکلسیم کلرید وغلظت اولیه ۲ و مس mg/L ۲ ثبت شد.