University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

Production of Thermostable Bioflocculant from Bacillus subtilis and Optimization of Flocculation Conditions

Husam Sabah Auhim

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

https://doi.org/10.32792/utq/utj/vol**20**/1/**4**

Abstract

Bacteria strain H7, which produces flocculating substances, was isolated from the soil of corn field at the College of Agriculture in Abu-Ghrib/Iraq, and identified as *Bacillus subtilis* by its biochemical /physiological characteristics. The biochemical analysis of the partially purified bioflocculant revealed that it was a proteoglycan composed of 93.2 % carbohydrate and 6.1 % protein. The effects of bioflocculant dosage, temperature, pH, and different salts on the flocculation activity were evaluated. The maximum flocculation activity was observed at an optimum bioflocculant dosage of 0.2 mL /10 mL (49.6%). The bioflocculant had strong thermal stability within the range of 30-80 °C, and the flocculating activity was over 50 %. The bioflocculant had the highest flocculating activity at alkaline conditions pH 10 (71%), and when many salts were used as cations, $ZnSO_4.7H_2O$, $MnCl_2$, and $CuSO_4$ enhanced flocculation activity at 89%, 80%, and 73% respectively.

Keywords: bioflocculant, Bacillus sp, flocculating activity, thermostable.

Introduction

Flocculation is an effective and convenient method of removing suspended solids, colloids, and cell debris. Generally, the flocculants were classified into three groups of flocculants: (i) inorganic flocculants such as aluminum sulfate and polyaluminum chloride; (ii) or organic synthetic flocculants such as polyacrylamide derivatives and polyethylene amine; and (iii) naturally occurring flocculants such as chitosan, sodium alginate, and microbial flocculants (Kurane *et al*.,1994; Xia *et al*.,2008). Inorganic and organic flocculating agents such as those mentioned above are frequently used both in water treatment and fermentation industries because of their strong flocculating activity and low cost. However, studies have shown that synthetic flocculating substances may cause health and environmental problems. For example, the acrylamide monomer is not only a neurotoxin and a strong human carcinogen but also non-degradable in nature (Kwon *et al.*,1996).On the other hand,

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

bioflocculant are essential polymers produced by some microorganisms during their growth, with their flocculating activity being dependent on the characteristics of the flocculants. These have special advantages such as safety, strong effect, biodegradability and harmlessness to humans and to the environment, which make them potentially suitable for application in drinking water and wastewater treatment, downstream processing, and fermentation processes (Salehizadeh and Shojaosadati,2001). The aim of this study was to isolate bacteria that produce bioflocculant compounds from soil and study the optimization of flocculating conditions. A series of experiments were then performed to characterization of bioflocculant.

Materials and Methods

Microorganism

The microorganisms were isolated using routine microbiological techniques from the soil; the isolates were maintained on a slant nutrient agar medium at 4 °C.

Growth Medium and Culture Conditions

The medium for a slant contained (per liter) 1 g yeast extract, 1 g beef extract, 2 g tryptone, 10 g glucose, 0.02 g $FeSO_4$ and 20 g agar (Xiong *et al.*,2010) The seed medium contained (per liter) 10 g glucose, 1 g peptone, 0.3 g $MgSO_4 \cdot 7H_2O$, 5 g K_2HPO_4, 2 g KH_2PO_4. The fermentation medium contained (per liter) (Zhang *et al.*,2007) 10 g glucose, 1 g peptone, 0.3 g $MgSO_4 \cdot 7H_2O$, 5 g K_2HPO_4, 2 g KH_2PO_4, initial pH of all media was adjusted to 7.2 with NaOH (1 M) and HCl (0.5 M). All media were prepared with distilled water and sterilized at 121°C /15 pounds for 20 min. All cultivations were done at 37°C.

Screening of the highest flocculant-producing isolates

Strains with different colony morphologies were selected and inoculated in 250-ml flasks containing 50 ml fermentation medium. The strains were incubated for 48 h at 37°C with shaking at 200 rpm. The flocculating activities of the culture broths were observed. The strain with the highest flocculating activity H7, was selected and then stored on a slant nutrient agar medium at 4°C for further research.

Production of bacterial bioflocculant

Strain H 7 from a slant nutrient agar medium was inoculated into a 250-ml flask containing 100 ml seed medium and cultivated at 37°C for 14 h at 200 rpm. Five milliliters of the culture was then transferred into another 250-ml flask containing 100 ml fermentation medium. The bioflocculant was produced by shaking the flask at 37°C and 200 rpm for 48 h. Cell-free supernatant was obtained by centrifugation at 10000 rpm for 15 min. The flocculating activity of the cell-free supernatant was determined. An uninoculated medium was used as a control.

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

Measurement of flocculating activity

Using a suspension of kaolin clay as test material, flocculating activity was determined according to Kurane *et al*,.(1986), as modified by Gao *et al*,. (2006). A suspension of kaolin clay (4 g/L) in deionized water at pH 7 was used as a stock solution for the subsequent assays. The following solutions were mixed in a test tube: kaolin clay suspension (9 mL), culture supernatant (0.1 mL) and 1% $CaCl_2$ (0.9 mL). A control in which the culture supernatant was replaced with deionized water was also included and measured under similar conditions. The final volume of all mixtures was made up to 10 mL with deionized water. The solutions were mixed gently and allowed to settle for 5 min. at room temperature. The optical density (OD) of the clarifying upper phase solution was measured at 550 nm using a UV-visible spectrophotometer (Varian, Australia), and the flocculating activity was determined as follows:

$$\text{Flocculating activity} = [(B - A)/B] \times 100\%$$

Where A and B are optical densities at 550 nm of the sample and control respectively.

Extraction and partial purification of bioflocculant.

Bioflocculant purification was achieved according to the modified method (Gong *et al.*, 2008). To purify the bioflocculant, the fermentation broth was centrifuged to remove cells (10000 rpm, 15 min). The supernatant was poured into two volumes of cold ethanol at 4 ºC to precipitate the bioflocculant. After 12 h, the resulting precipitate was collected by centrifugation at 5000 rpm for 30 min and redissolved in water. After two steps, the precipitate was dehydrated at 40 ºC, and the bioflocculant was obtained.

Analysis of partially purified bioflocculant

The total sugar content of bioflocculant was determined by a phenol-sulphuric acid method using glucose as a standard solution (Dubois *et al.*,1956). The total protein content of purified bioflocculant was determined by Lowry's method using Bovine Serum Albumin (BSA) as a standard (Lowry *et al.*,1951)

Effect of bioflocculant dosage, temperature, pH, and many salts on bioflocculant activity

Various amounts (0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 mL) of the cell-free supernatant were added to a test tube containing (9 mL) of kaolin clay (4 g/L) and (0.9 mL) of 1% $CaCl_2$. To examine thermal stability of the bioflocculant, the cell-free supernatant after 10 min at various temperatures (30-90°C) was used to measure the flocculating activity at room temperature. The effects of pH and many salts on flocculating activity were examined. HCl and NaOH solutions were used to adjust the pH of the kaolin suspension in three groups (3, 7 and 10 pH).

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

Solutions (1 wt %) of NaCl, $MnCl_2$, $MgSO_4$, $ZnSO_4.7H_2O$, and $CuSO_4$ were used as cations sources in replacing $CaCl_2$ solutions to measure flocculating activity. The flocculating activity was determined as previously described.

Results and Discussion

Isolation and identification of the bioflocculant producing strain

In this study, a total of 12 bioflocculant-producing strains have been isolated from the agricultural soil. According to Peter *et al*., (1986), all those isolates showed *Bacillus* sp characteristics, either physiological or morphological criteria, including positive Gram reaction, endospore appearance, and catalase enzyme activities. Strain H7 showed the highest flocculating activity in kaolin suspension and was thus chosen for further research. Strain H7 was identified as *Bacillus subtilis* by its biochemical and physiological characteristics, as shown in Table 1.

Table 1. Characteristics of isolated strain H7

Characteristics	**Result**
Gram stain	+
Shape Rod	Rod
Endospores produced	+
Spore location	Subterminal
Motile	+
Characteristics	**Result**
Physiological characteristics	
Catalase test	+
Voges-Proskauer test	+
Gas from glucose	-
Urease production	+
Nitrate reduced to nitrite	+
Citrate utilization	+
Casein and starch hydrolysis	+
Gelatin liquefaction	+
Indole formation	-

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

Acid from	
D-glucose	+
L-Arabinose	+
D-Xylose	+
D-Mannitol	+
Culture characteristics	
Anaerobic growth	-
Growth in 7% NaCl	+
Growth on pH 5.7	+
Growth at 50°C	+
Growth at 55°C	-

+: positive, -: negative

Many *Bacillus* sp that produced bioflocculants were studied, such as: *Bacillus coagulans* (Salehizadeh *et al*., 2000) ; *Bacillus licheniformis* (Shih *et al*.,2001); *Bacillus firmus* (Salehizadeh and Shojaosadati,2002).; *Bacillus mucilaginosus* (Deng *et al*.,2003); *Bacillus megaterium* (Zheng *et al*.,2008) and *Bacillus subtilis* (Patil *et al*.,2009).

Characterization of bioflocculant

Analysis of partially purified bioflocculant

The biochemical analysis of the partially purified bioflocculant revealed that it was a proteoglycan composed of 93.2 % carbohydrate and 6.1 % protein. Xiong *et al*. [6] reported that the bioflocculant from *B. licheniformis* CGMCC 2876 was comprised of 89 % (wt/wt) carbohydrate and 11% (wt/wt) protein. (Patil *et al*.,2009) demonstrated that the bioflocculant from *Bacillus subtilis* was composed of 94.3% polysaccharide and 5.7% protein.

Effect of bioflocculant dosage on flocculation activity

Figure 1 shows the relationship between the flocculant dosages and flocculation activities. When the cell-free supernatant in kaolin suspension (4.0 g/L) was tested in the dosage range of (0.05, 0.1, 0.15, 0.2,0.25, and 0.3 mL), it was apparent that the flocculation activity increased proportionally to the flocculants dosage of 0.05 to 0.2 mL and was highest at 0.2 mL(49.6%) then the flocculation activity was decreased slightly. These results could be clarified as follows: a) The incomplete dispersion of excess polysaccharide, only the kaolin particles around the polysaccharides participated in the flocculation reaction. Therefore, other kaolin

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

particles did not participate in the reaction (Yokoi *et al*.,1997), and b) The excess polysaccharide was oversaturated on many binding sites of the surface of kaolin particles; thus the attractive force of the other particles was reduced, and the flocculation activity decreased (Kwon *et al*.,1996). Thus, either the deficiency or excess amount of polysaccharide and kaolin clay decreased or even prevented the flocculation activity (Lee *et al*.,1995).

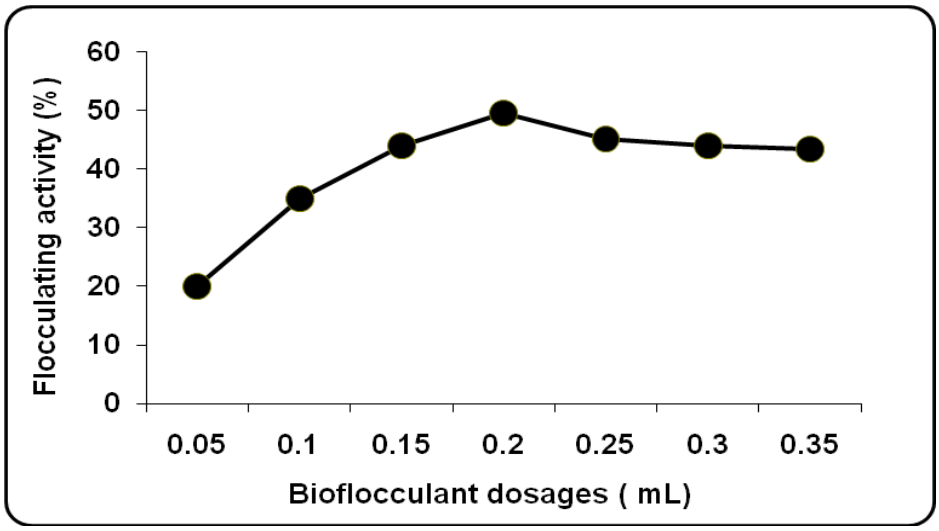


Figure 1. Effect of bioflocculant dosages on flocculating activity.

Thermal stability of the bioflocculant

The bioflocculant had strong thermal stability within the 30-80 °C range, and the flocculating activity was over 50 %, as shown in Figure 2. The lower flocculating activity of the bioflocculant at higher temperatures above 90°C may be due to the breaking down of the polysaccharide chain, which led to the low potential to form bridges with the kaolin particles (Patil *et al*.,2009; Liu *et al*.,2010) reported bioflocculant produced by *Bacillus subtilis* showed heat stability at 97 °C for 10 minutes. Some researches indicate that the heat resistance of bioflocculant is consistent with the general understanding that flocculants rich in polysaccharides have better thermal resistance than those of proteins and nucleic acids (Kurane *et al.*, 1986; Zhang *et al*.,2012).This indicates the thermostability of bioflocculant at a wide range of temperatures, which is useful in many applications using high temperatures.

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

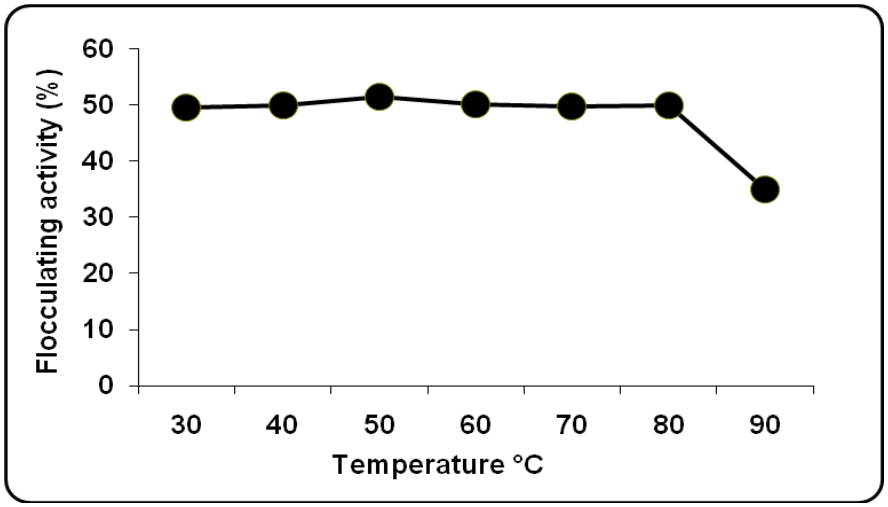


Figure 2. Effect of temperature on flocculating activity

Effect of pH on flocculating activity

The pH of the solution is also a key factor in flocculation and thus effectively influences the flocculation process (Bouchotroch *et al*.,2001). Figure 3 shows the effects of pH on flocculating activity. The flocculating activity was found to be highest at alkaline conditions (71%), and some bioflocculant have more binding sites and stronger Vander Waals forces than traditional flocculating agents, strengthening their bridging ability between suspended kaolin clay particles. However, under acid conditions, the ionization of COOH in bioflocculants will be blocked, restraining the bridging actions. While under an alkaline condition, $^{-}$COOH will be ionized into $COO^{=}$, and OH^{-} will be increased, both of which can promote the flocculating efficiency of bioflocculant (Zhang *et al*.,2010).

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

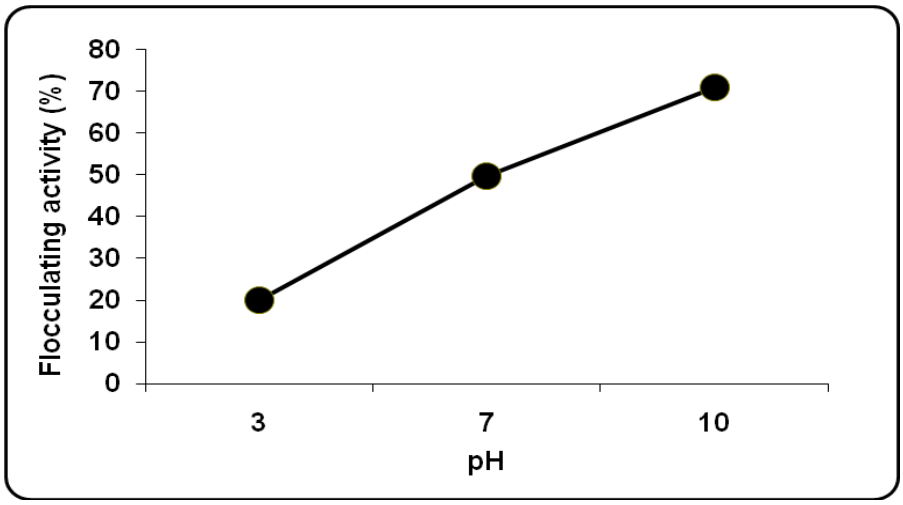
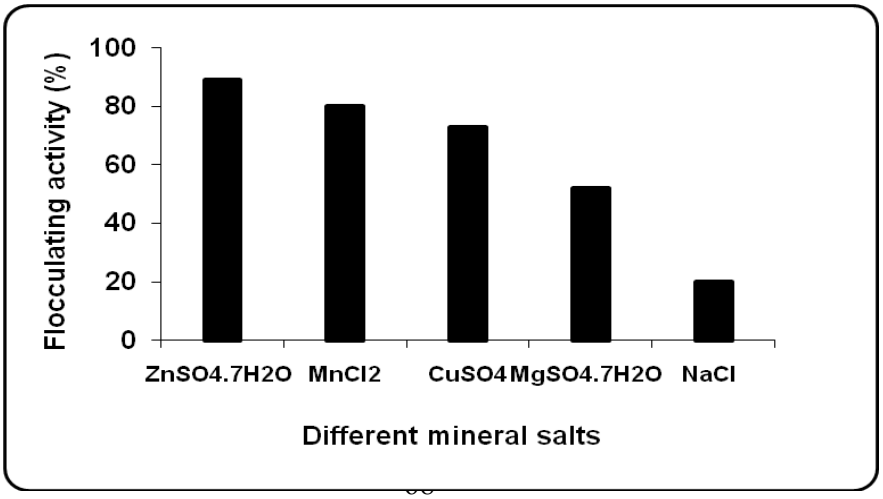


Figure 3- Effect of pH on flocculating activity

Effect of different salts on flocculating activity

Metal ions either stimulate or inhibit flocculating activity (Li *et al.*,2009; Liu *et al.*,2010). Among the mechanisms proposed for stimulation are: (1) neutralization and stabilization of the residual charge of a functional group on the bioflocculant by the metal ions (Kwon *et al.*,1996) and (2) increase in ionic strength of the suspension solution as a result of the addition of metal ion; thereby, decreasing electrostatic forces of the suspended particles (Wang *et al.*,2011). Results showed that $ZnSO_4.7H_2O$, $MnCl_2$, and $CuSO_4$ enhanced flocculation activity 89%,80%, and 73%, respectively, when compared to the $MgSO_4.7H_2O$ and NaCl 52% and 20%, respectively. Figure 4.



University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

Figure 4- Effect of different salts on flocculating activity

Conclusions

The bioflocculant produced by Strain H 7 showed good flocculating activity for kaolin suspension. Chemical analyses indicated the bioflocculant to be proteoglycan composed of 93.2 % carbohydrate and 6.1 % protein; bioflocculant had strong thermal stability and highest flocculating activity at the alkaline condition which is useful in many applications using high temperature and alkaline conditions**,** bioflocculant showed highest flocculating activity when used $ZnSO_4.7H_2O$ as cation so that this bioflocculant can be used in treatment wastewater contaminated with Zn^{+2} ions.

Acknowledgments

I am grateful to the University of Baghdad, College of Science, Department of Biology, for providing the possible laboratory materials and equipment during the research period**.**

References

Bouchotroch, S., Quesada, E., Del Moral, A., Llamas, I. and Béjar, V. (2001). *Halomonas maura sp*. nov., a novel moderately halophilic, exopolysaccharide-producing bacterium. Int. J. Syst. Evol. Microbiol. 51:1625–1632.

Deng, S. B., Bai, R. B., Hu, X. M. and Luo, Q. (2003). Characteristics of abioflocculant produced by *Bacillus mucilaginosus* and its use in starch wastewater treatment. Appl. Microbiol. Biotechnol. 60:588–593.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350–356.

Gao, J., Bao, H.-Y., Xin, M.-X., Liu, Y.-X., Li, Q. and Zhang, Y.-F. (2006). Characterization of a bioflocculant from a newly isolated *Vagococcus sp*. W31. J. Zhejiang Univ. Sci. B . 7,186–192.

Gong, W., Wang, S., Sun, F., Liu, X.W., Yue, Q.Y. and Gao, B.Y. (2008). Bioflocculant production by culture of *Serratia ficaria* and its application in wastewater treatment. Bioresour. Technol. 99:4668–4674.

Kurane, R., Hatamochi, K., Kiyohara, T., Hirao, M. and Taniguchi, Y. (1994). "Production of a bioflocculant by *Rhodococcus erythropolis* S-1 grown on alcohols," Biosci. Biotechnol. Biochem. 58: 428–429.

Kurane, R., Takeda, K. and Suzuki, T. (1986). Screening for and characteristics of microbial flocculants. Agr. Biol. Chem. 50: 2301-2307.

Kwon, G. S., Moon, S. H., Hong, S. D., Lee, H. M., Kim, H. S. and OH, H. M. (1996). A novel flocculant biopolymer produced by *Pestalotiopsis sp*. KCTC 8637P. Biotech. Lett. 18: 1459–1464.

Lee, S. H., Lee, S. O., Jang, K. L.and Lee, T. H. (1995).Microbial flocculant from *Arcuadendron sp*. TS-49. Biotech. Lett., 17: 95–100.

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

Li, Z., Zhong, S., Lei, H., Chen, R., Yu, Q. and Li, H.L. (2009). Production of a novel bioflocculant by *Bacillus licheniformis* X14 and its application to low temperature drinking water treatment. Bioresour. Technol. 100:3650–3656.

Liu, W., Wang, K., Li, B., Yuan, H. and Yang, J. (2010). Production and characterization of an intracellular bioflocculant by *Chryseobacterium daeguense* W6 cultured in low nutrition medium. Bioresour.Technol., 101: 1044–1048.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with Folin Phenol reagent. J. Biol. Chem. 196: 256–275.

Patil S.V, Bathe G.A, Patil A.V, Patil R.H and Salunkea,B.K. (2009).Production of bioflocculant exopolysaccharide by *Bacillus subtilis*. Adv. Biotech. 7: 14-17.

Peter, H.A.S, Nicholas, S.M., Sharpe, M.E. and Holt, J.G. (1986). Bergey's Manual Of Systematic Bacteriology, Williams & Wilkins, Baltimore, USA, pp 1104-1139.

Salehizadeh, H. , Shojaosadati S. A. (2002). Isolation and characterization of a bioflocculant produced by *Bacillus firmus*. Biotechnol. Lett. 24: 35–40.

Salehizadeh, H. , Shojaosadati, S.A. (2001). Extracellular biopolymeric flocculants-recent trends and biotechnological importance. Biotechnol. Adv. 19: 371–385.

Salehizadeh, H, Vossoughi M. and Alemzadeh I. (2000). Some investigations on bioflocculant producing bacteria, Biochem. Eng. J. 5: 39-44.

Shih, I.L., Van, Y.T., Yeh, L.C., Lin, H.G. and Chang, Y.N. (2001). Production of a biopolymer flocculant from *Bacillus licheniformis* and its flocculation properties. Bioresour. Technol. 78: 267-272.

Wang, L., Ma, F., Qu, Y., Sun, D., Li, A.; Guo, J. and Yu, B. (2011). Characterization of a compound bioflocculant produced by mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6.World J. Microbiol. Biotechnol. 27: 2559–2565.

Xia, S., Zhang, Z., Wang, X., Yang, A., Chen, L., Zhao, J., Leonard, D. and Jaffrezic-Renault, N. (2008). Production and characterization of bioflocculant by *Proteus mirabilis* TJ-1. Bioresour. Technol. 99: 6520–6527.

Xiong, Y., Wang, Y., Yu, Y., Li, Q., Wang, H., Chen, R. and He, N. (2010). Production and characterization of a novel bioflocculant from *Bacillus licheniformis*. Appl. Environ. Microbiol. 9: 2778-2782.

Yokoi, H., Yoshida, T., Mori, S., Hirose, J., Hayashi, S. and Takasaki, Y. (1997). Biopolymer flocculant produced by an *Enterobacter sp*. Biotech. Lett. 19: 569–573.

Zhang, Z., Lin, B., Xia, S., Wang, X. and Yang, A. (2007). Production and application of a novel bioflocculant by multi-microorganism consortia using brewery wastewater as carbon source. J. Environ. Sci. 19: 667–673.

Zhang,C., Cui,Y. and Wang,Y. (2012). Bioflocculant produced from bacteria for decolorization ,Cr removal and swine wastewater application. Sustain. Environ. Res. 22: 129-134.

University of Thi-Qar Journal

ISSN (print): 2706- 6908, ISSN (online): 2706-6894

Vol.20 No.1 Mar 2025

Zhang,Z,Xia,S.,Zhao,J. and Zhang,J.(2010). Characterization and flocculation mechanism of high efficiency microbial flocculant TJ-F1 from *Proteus mirabilis*. Colloids Surf. B. Biointerfaces, 75:247-251.

Zheng, Y., Ye, Z.-L., Fang, X.-L., Li, Y.-H. and Cai, W.-M. (2008). Production and characteristics of a bioflocculant produced by *Bacillus sp*. F19. Bioresour. Technol. 99: 7686–7691.

انتاج الملبد الحيوي الثابت حرارياً من جنس *Bacillus subtilis* ودراسة تحسين ظروف التلبد

حسام صباح أوهيم

قسم علوم الحياة - كلية العلوم - جامعة بغداد - بغداد - العراق

الخلاصة

عزلت السلالة البكتيرية H7 المنتجة للمواد الملبده من تربة حقول الذرة في كلية الزراعة في ابو غريب وقد شخصت هذه السلالة على انها احدى سلالات *Bacillus subtilis* اعتماداً على الصفات الكيموحيوية والفسيولوجية , اظهرت نتائج التحليل الكيميائي للملبد المنقى جزئياً بانه بروتوكلايكان حيث يتكون من 93.2 % كاربوهيدرات و 6.1 % بروتين . درست خصائص الملبد الحيوي اعتماداً على فعالية التلبد لعالق الكاؤلين بوجود 1% من كلوريد الكالسيوم (مصدر الشحنة الموجبة) لتحسين عملية التلبد, اظهرت النتائج ان الملبد الحيوي اعطى فعالية تلبد جيدة لعالق الكاؤلين (49.6%) عند حجم 0.2 مل لكل 10 مل , كما اظهر الملبد الحيوي ثباتية عاليه على مدى واسع من درجات الحرارة تراوحت بين 30 – 80 مئوية وكانت فعالية التلبد فوق 50 %, كان الاس الهيدروجيني الافضل لعملية التلبد هو 10 وعند دراسة تأثير الاملاح (مصدر الشحنة الموجبة)على فعالية التلبد بدلا من كلوريد الكالسيوم اظهرت النتائج بان ملح كبريتات الزنك اعطت اعلى فعالية تلبد 89 % يليه ملح كلوريد المنغنيز 80% واخيراً ملح كبريتات النحاس 73%.