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THE POTENTIAL OF COBALT BIOACCUMULATION FROM AQUEOUS SOLUTIONS BY USING THE EXOPOLYSACCHARIDE PRODUCING BACTERIUM, *Xanthomonas Campestris*

Ashour, Eman H.
Microbiology Dept., Faculty of Agriculture, Mansoura University, Mansoura, Egypt. Email: eashour@mans.edu.eg

ABSTRACT

The removal of cobalt (as a toxic metal of high environmental priority due to its toxicity) from dilute aqueous solutions has been studied in the present work. An exopolysaccharide (EPS) producing bacterium, *Xanthomonas campestris* was tested for its Co tolerance. It not only could grow on medium containing cobalt as high as 100 mg l^{-1} but also showed enhanced exopolysaccharide production. 68% of cobalt was accumulated by both bacterial cells and EPS The bioaccumulation characteristics for cobalt with different types of biosorbents were studied. The data performed that the living cells of *X. campestris* had higher cobalt bioaccumulation capacity than nonliving cells, in addition, the mixtures of either living or nonliving cells with EPS lead to enhancement of cobalt removal. The cobalt bioaccumulation of the *X. campestris* was influenced by the initial pH of solution, initial metal ion concentration, biosorbent type/concentration, and biosorbent pretreatment. The results of this study demonstrated that the living cells of this bacterial strain could remove cobalt from solution with relatively high efficiency, which reached to 80 mg cobalt g^{-1} biosorbent.

INTRODUCTION

Heavy metal pollution represents an important problem due to its toxic effect and accumulation throughout the food chain which leads to serious ecological and health hazards as a result of their solubility and mobility. Metal remediation through common physico-chemical techniques is expensive and not eco-friendly. Hence, biotechnological approaches have received a great deal of attention as an alternative tool in the recent years.

The ability of metal uptake by biomass (known as biosorption or bioaccumulation) is usually classified into three categories (Gadd 1988 and Volesky and Holan 1995): (1) cell surface binding, (2) intracellular accumulation and (3) extracellular accumulation. Being metabolism independent, the cell surface binding can occur in either living or inactivated microorganisms, whereas the intracellular and extracellular accumulation of metals are usually energy-driven processes, and thus can take place only in living cells.

Applicability of growing bacterial, fungal, algal cells for metal removal and the efforts directed towards cell/process development to make this option technically and economically viable for the treatment of metal rich effluents have been reviewed by Malik (2004), Zouboulis *et al.* (2004) and Pal *et al.* (2006).

The application of living bacteria (Diels *et al.* 1999 and Gadd 2000) and biopolymers (Gutnick and Bach 2000) has been recently incorporated into the concept of biosorption. Biosorbents may be viewed as natural ion exchange materials that primarily contain weakly acidic and basic groups (Kratochvil and Volesky 1998).

The need for economical, effective and safe methods for removal of heavy metals from wastewater has directed attention to extracellular polysaccharide or exopolysaccharide (EPS) produced by algae, bacteria, fungi and yeast (Volesky and Holan 1995 and Salehizadeh and Shojaosadati, 2001). Few reports on metal chelation by bacterial extracellular polysaccharide are available today. The adsorption of heavy metals by exopolysaccharide is non-metabolic, energy independent and can be caused by interaction between metal cations and negative charge of acidic functional groups of exopolysaccharide (Kim *et al.* 1996). Exopolysaccharides are also recommended as surface-active agents for heavy metal removal (Kaplan *et al.* 1987).

A polysaccharide from *Bacillus firmus* is reported to remove metal ions (like Pb, Cu and Zn) from aqueous solution (Salehizadeh and Shojaosadati 2003). *Enterobacter cloaceae*, an exopolysaccharide producing marine bacterium, was tested for chromium accumulation (Iyer *et al.* 2004, 2005).

In this study, the toxicity of cobalt and bioaccumulation capacity of *Xanthomonas campestris* was investigated. The use of cells together with exopolysaccharide (EPS) as a biomaterial for metal bioaccumulation was tested. The effects of physical factors (*i.e.*, biosorbent type, biosorbent pretreatment, biosorbent densities, initial pH of solution and initial cobalt concentration) on cobalt bioaccumulation were also studied to further evaluate the feasibility of applying this strain in particular heavy metal removal process.

MATERIALS AND METHODS

Bacterial strain and media

The bacterium used in this study was *Xanthomonas campestris* NRRL B-1459. This strain has been previously used (Ashour *et al.* 2000) for overproduction of exopolysaccharide, xanthan. It was grown aerobically in YPGM medium (Rajeshwarl *et al.* 1995) at pH 7.0, which contained (g/L): 20 of glucose; 5 of peptone; 5 of KH_2PO_4; 2 of $MgSO_4$-$7H_2O$; and 2 of yeast extract.

Effect of cobalt on bacterial growth and cobalt content

X. campestris NRRL B-1459 was grown for 16 h in YPGM medium (Rajeshwarl *et al.* 1995) and used as inoculum (approximately 10^8 cells ml^{-1}). One milliliter of inoculum was added to 100 ml of sterile YPGM medium in 500 cm^3 Erlenmeyer flasks containing different concentrations of Co (as $CoCl_2$), i.e., 25, 50, 75, 100 ppm. Each of the sets was prepared in duplicates. One set of medium without Co was also inoculated and kept as control. After inoculation the flasks were incubated at 30°C on an orbital shaker at 120 rpm for 72 h. For determination of the cell dry weight, the culture broth was

centrifuged (at 10,000 rpm for 20 min) and cell pellets produced after centrifugation were dried at 105 °C for 24 h. The traditional method of alcohol (isopropanol) precipitation of the supernatant was used for the recovery of the exopolysaccharide. Dry weight of the exopolysaccharide and bacterial biomass were measured. In both bacterial cells and exopolysaccharide, the cobalt content was measured.

Bioaccumulation experiments

1-Preparation of biosorbents

Living cells (LC): The cells were harvested by centrifugation (10,000 rpm and 20 min) from broth culture (72 h-old) and washed twice with distilled water.

Nonliving cells (NLC): The broth culture (72 h-old) was inactivated by boiling (100°C, 15 min) before being harvested by centrifugation. The living and nonliving cells were harvested by re-centrifugation.

Exopolysaccharide (EPS): To precipitate exopolysaccharide in the supernatant (after separating the living cells), two volumes of cold ethanol (4°C) were added with agitation vigorously and held at 4°C for 4 h (Tago and Aida 1977). Precipitated exopolysaccharide (as well as living and nonliving cells) were collected and air dried in a desiccator overnight.

2-Effect of biosorbent type

From each of biosorbent types (LC, NL, EPS, LC-EPS and NLC-EPS), 0.1 g dry weight were mixed with 100 ml of cobalt solution (100 ppm) in 500 cm^3 flasks at pH 6. 0.1 N HCl or 0.1 N NaOH was used for pH adjustment. Then the flasks were agitated on the orbital shaker (120 rpm) at room temperature for 120 min. At desired intervals, samples were filtered through Whatman 0.45 µm membrane filters and the resulting filtrate was used to estimate the residual cobalt concentration. In order to account the effect of filter paper on cobalt adsorption, a separate set of control experiment was done with the same conditions for all experiments.

3-Effect of cobalt concentration

Biosorbents (1.0 g dry weight l^{-1}) were suspended in aqueous solutions containing Co at various initial concentrations at pH 6. The mixtures were incubated for 1h.

4-Effect of biosorbent densities

Various amounts of biosorbents were added at concentrations of 0.25 to 2.0 g dry weight per liter to test solution containing 100 ppm of cobalt and the mixtures were incubated for 1h.

5-Effect of pH

Biosorbents (1.0 g dry weight l^{-1}) were suspended in aqueous solutions containing 100 ppm of cobalt at various pH values. The pH value of the mixtures was adjusted by 0.1 N HCl or 0.1 N NaOH then followed the same procedure.

6-Effect of competing ions

Biosorbents (1.0 g dry weight l^{-1}) were incubated in aqueous solutions containing 100 ppm of cobalt plus 100 ppm of chloride salts of selected metal ions. Controls received 100 ppm of cobalt only. The mixtures were incubated for 1h.

7-Effect of cell pretreatments

Aliquots of prepared biosorbents were exposed to the following physical or chemical treatments immediately prior to cobalt-removal assays. All treatments were performed with biosorbents suspended at a final concentration of 1 g dry weight per liter. For heat treatment, biosorbents were incubated at 100°C for 10 min. For acid and alkali treatments, biosorbents were incubated for 10 min with 50 mM of either HCl or NaOH. For enzyme treatment, biosorbents were incubated with 1 mg of trypsin per ml at 30°C for 1 h. For ether treatment, biosorbents were suspended in diethyl ether and incubated at 22°C for 10 min. Controls were kept in ddH_2O at 10°C for I h. Controls and treated samples were harvested by filtration and washed three times. The remainder of the preparation was tested immediately for Co-removal (100 ppm cobalt chloride solution, I h incubation).

Cobalt Analysis

The concentration of bioaccumulated (bounded) cobalt was determined for dried samples of the bacterial cells and exopolysaccharide. The samples were digested using nitric acid. The digested mixture was then centrifuged and metal in the supernatant was assayed. The concentration of unbounded cobalt in the biosorption medium was determined in the filtrate that was adjusted to pH 2.0 with nitric acid before analysis. Then, cobalt ions concentration was measured by the use of an atomic absorption spectrophotometer, Perkin Elmer Analyst 3100 with a specific lamp for cobalt and at specific wavelengths. The amount of cobalt bioaccumulated by the biosorbents was calculated by using the following equation (Salehizadeh and Shojaosadati 2003): $Q = V (C_0 - C) / W$

Where Q is the amount of metal ions adsorbed onto the unit amount of the biosorbents (mg/g), V is the volume of aqueous phase (l^{-1}), W is the mass weight of biosorbent (g), C_0 and C are the concentration of metal ions in initial solution (mg l^{-1}) and after biosorption, respectively .

RESULTS AND DISCUSSION

Effect of cobalt on the bacterial growth and cobalt bioaccumulation

Data represented in Table (1) revealed that growth of *X. campestris* NRRL B-1459 was observed in all the treatments (cobalt free as control and those containing Co) after 72 h of incubation. An increase in concentration of cobalt caused an increase in cells production (which can be seen by the increase in dry weight of the bacterial biomass). A similar pattern was also noticed in case of polysaccharide production. This indicated that the culture was not only tolerant to cobalt but its presence stimulated exopolysaccharide production. It was also observed remarkable bioaccumulation amount of cobalt by the bacterial biomass and the exopolysaccharide with an increase in concentration of cobalt in the medium. The total cobalt removal by the cells and exopolysaccharide was 64, 68, 63 and 61% in the presence of 25, 50, 75 and 100 ppm of cobalt, respectively. These results are in harmony with data of Iyer *et al.* (2004), where both the biomass and the exopolysaccharide accumulated about 60-70% chromium.

Table 1. Influence of cobalt on dry weight and cobalt content in *X. campestris* NRRL B-1459

Co concn. (ppm)	Bacterial biomass			Exopolysaccharide			Total Co removal (%)
	Dry weight (g/100 ml)	Co content (mgg^{-1})	Co removal (%)	Dry weight (g/100 ml)	Co content (mgg^{-1})	Co removal (%)	
0	0.1862	ND	0	0.4202	ND	0	0
25	0.2375	2.427	23.06	0.6136	1.675	41.11	64
50	0.2646	4.596	24.32	0.7475	2.946	44.05	68
75	0.2080	8.250	22.88	0.6092	5.002	40 63	63
100	0.1651	13.349	22.04	0.5043	7.733	39.00	61

ND, non-detectable

The factors affect the Bioaccumulation process

Biosorbent type: Two types of biosorbents in addition to exopolysaccharide were prepared and examined. The nonliving cells, NLC, are chemically stable and easy to handle, but some of the metal binding sites may be destroyed during the boiling. In contrast, the chemical properties of living cells, LC, may vary with time and are expecting to have higher metal-binding capacity than nonliving cells do.

Time-course profiles for the bioaccumulation of cobalt by different types of biosorbents (LC, NLC, EPS, LC-EPS and NLC-EPS) are shown in Fig. 1. In all cases, the metal bioaccumulated rapidly during the first 30 min, slowly removed during the next 30 min and remained nearly constant after 2 h of biosorption, suggesting that the bioaccumulation is fast and reaches saturation within 2 h. The cobalt bioaccumulation for living cells (LC) and living cells + exopolysaccharide (LC-EPS) is apparently higher than that for nonliving cells (NLC) as well as exopolysaccharide (EPS) and their mixture. After 60 min, a maximum cobalt bioaccumulation value was 80.19 mg Co g^{-1} dried living cells (LC). This value obviously is low when compared with those reported by Salehizadeh and Shojaosadati (2003), who found that *Bacillus firmus*' polysaccharide has recorded a maximum value of uptake of 1103, 860, and 722 mg g^{-1} for Pb, Cu, Zn, respectively.

Decrement of cobalt bioaccumulation by using of nonliving cells may be attributed to the effect of heating, which may cause destroying or loss some of metal binding sites, resulting in the decrease in metal bioaccumulation efficiency for the nonliving cells. The other possibility is that intracellular accumulation of metal ions may occur in living cells, resulting in the enhancement in metal bioaccumulation efficiency.

Biosorbent densities: Metal bioaccumulation can be initiated by changing initial cells concentration. Data shown in Fig. 2 indicate that metal bioaccumulation was inversely proportional to the used biosorbent densities. The decrease of metal bioaccumulation might be attributed to metal concentration shortage in solution (Esposito *et al.* 2001). The results reveal that bioaccumulation of cobalt with NLC-EPS was not pronounced affected by the cells densities increment comparing with Co adsorption capacity of LC-EPS. The amount of cobalt bioaccumulated deceased from 101.23 to 32.12 mg g^{-1} LC-EPS biosorbent when concentration of used biosorbent increased from 0.25 to 2.5 g l^{-1}. Though, Taniguchi *et al.* (2000) reported that reported

that the amount of Zn(II) adsorbed by cells decreased from 32 to 8 mg g^{-1} dry cells when the added cell mass was increased from about 2 to 52 g l^{-1}, and they proposed that such a trend was simply due to dilution of zinc with the added cells.

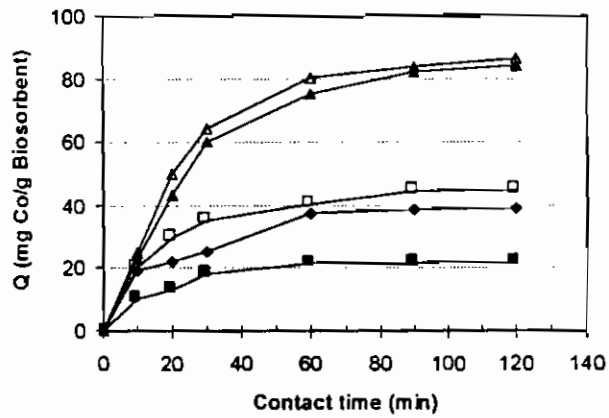

Fig. 1. Time-course profiles of cobalt bioaccumulation by different types of biosorbents of *X. campestris*. LC (▲); NLC (■); EPS (♦); LC-EPS (△) and NLC-EPS (□)

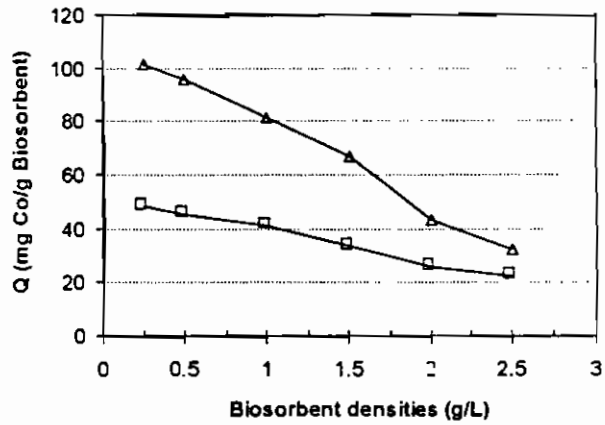

Fig. 2. Biosorbent densities and cobalt bioaccumulation of *X. campestris* LC-EPS (△) and NLC-EPS (□).

Biosorbent pretreatments: The physical and chemical treatments tested had various effects on Co bioaccumulation with *X. campestris* LC-EPS (Table 2). Alkali pretreatment enhanced Co bioaccumulation, while acid pretreatment was inhibitory. The results of ether and trypsin treatments suggest that lipids and phospholipids are mostly not involved in cobalt bioaccumulation with this organism. This last finding is in agreement with results presented by Faison *et al.* (1990). Evidence for the existence of intracellular Co-binding sites in *X. campestris* LC-EPS is provided by the results of chemical pretreatments.

Table 2. Effect of cell pretreatments on cobalt bioaccumulation by *X. campestris* LC-EPS.

Treatment	Target component	Relative removal (%)
NaOH	Nonspecific	129
Control	-	100
Ether	Lipids	91
Trypsin	Protein	82
Boiling (100°C)	Nonspecific	73
HCl	Nonspecific	12

Initial cobalt concentration: The metal bioaccumulation of LC-EPS and NLC-EPS biosorbents (constant densities) after 120 min gradually increased with increment of the initial concentration of metal ions and reached almost a saturated value at 500 mg l^{-1} (Fig. 3). When the initial cobalt concentration was increased from 12.5 to 400 mg l^{-1}, the bioaccumulation by *X. campestris* LC-EPS and NLC-EPS increased from 11.11 to 140.48 mg g^{-1} and from 9.58 to 95.23 mg g^{-1}, respectively.

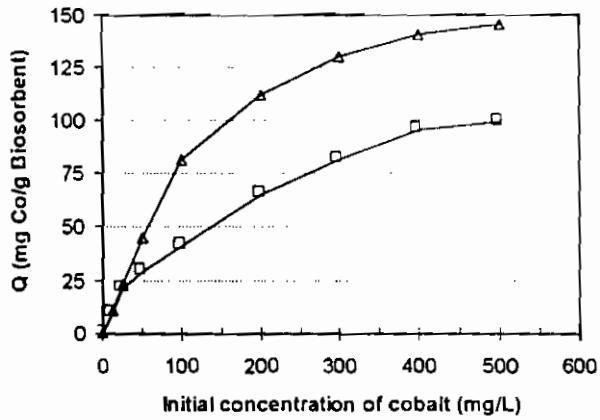

Fig. 3.Influence of initial metal concentration on cobalt bioaccumulation by *X. campestris* LC-EPS (△) and NLC-EPS (□).

pH values: The wastewaters are characterized by substantial variations in pH values and hence the initial pH of the solution is an important factor to be considered during biosorption studies (Awadalla and Pesic 1992 and Zouboulis *et al.* 2004). Moreover, It is well-known that metal ion adsorbed on both non-specific and specific sorbent is pH dependent (Churchill *et al.* 1995). The medium' pH affects the solubility of metal ions and the ionization state of the functional groups (i.e., carboxylic, phosphate and amino groups) on the bacterial cell surface. Values of pH above 8 were not studied because cobalt removal by hydroxide formation may begin to interfere with cobalt removal by biosorption above pH 8 (Awadalla and Pesic 1992). Data illustrated in Fig. 4 shows that bioaccumulation of cobalt ions by used biosorbents first increased with pH, and maximum cobalt ions bioaccumulation occurred at pH 5.0 and 6.0. The bioaccumulation of cobalt ions with the two types of biosorbents (LC-

EPS and NLC-EPS) increased as initial pH of solution increases towards acidic and then decline with further increase in pH. With increase in pH the hydronium ion concentration decreased leading to lesser competition for the active sites with cobalt ions, which resulted in maximum cobalt uptake at pH 7. However, the decrease of the bioaccumulation at alkaline pH might be due to the formation of soluble salt of cobalt which is predominant at higher pH values (Awadalla and Pesic 1992). Similar observations were reported for other biomass (Awadalla and Pesic 1992 and Suhasini *et al.* 1999).

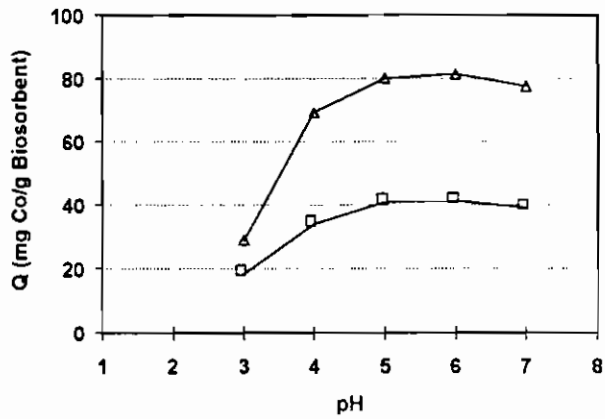

Fig. 4. Effect of initial pH on cobalt bioaccumulation by *X. campestris* LC-EPS (△) and NLC-EPS (□).

Presence of other ions: Bioaccumulation is mainly used to treat wastewater where more than one type of metal ions would be present; the removal of one metal ion may be influenced by the presence of other metal ions. Aqueous solution containing 100 mg l^{-1} of each ion was incubated with *X. campestris* LC-EPS for 120 min. The process of cobalt bioaccumulation is inhibited in the presence of other ions (Fig. 5). Both monovalent and divalent ions could be substituted for bound cobalt. Divalent ions replaced Co somewhat more effectively than did monovalent ions.

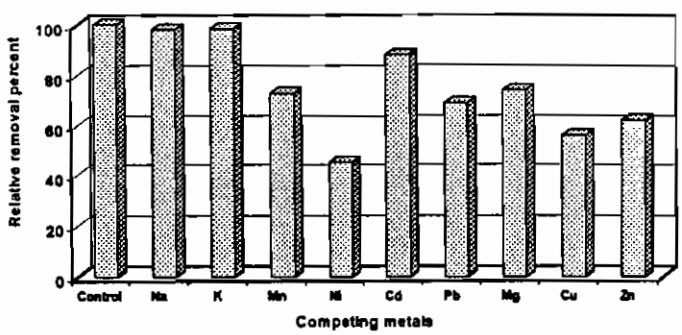

Fig. 5. The inhibition effect of other metal ions (in ration 1:1) on cobalt bioaccumulation by *X. campestris* LC-EPS.

Ni was the most competitive divalent ions forward Co ions, where the amount of cobalt bounded in the presence of nickel was only 45 % of the control. These effects were published by other researchers (Salehizadeh and Shojaosadati 2003 and Ozdemir *et al.* 2005).

It could be concluded that the aim of this work was to find the bioaccumulation of selected one of the bacteria producing the most EPS for removal of cobalt ions. Experiments were performed as a function of biosorbent type, pH, initial biosorbent/metal ion concentration and contact time. The obtained results showed that *X. campestris'* cells and EPS were good biosorbents for cobalt ions and had high bioaccumulation yields for the treatment of wastewater containing such metal, cobalt.

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إمكانية استخدام بكتيريا زانثوموناس كامبستريز المنتجة للسكريات العديدة لتجميع الكوبلت حيويا من الاوساط المائية

إيمان حسين عاشور
قسم الميكروبيولوجى – كلية الزراعة - جامعة المنصورة- المنصورة – مصر

تعتبر المعادن الثقيلة من أخطر المواد التي تلوث التربة والماء ، ومن أهم مصادر هذا التلوث مخلفات ونفايات المصانع وصهر المعادن واحتراق الفحم وعوادم السيارات ومبيدات الآفات. تكمن خطورة التلوث بالمعادن الثقيلة فى أنها ذات تأثير سلبى على البيئة ، على الرغم من قلة تركيزها احيانا فى مصادر التلوث إلا أن تراكمها فى أجسام الكائنات الحية بتركيزات عالية تسبب تأثيرات سلبية على تلك الكائنات وربما قد تنتقل فى نهاية المطاف الى الإنسان ضمن السلسلة الغذائية. وعليه يوصى الان بالمعالجة الحيوية كوسيلة آمنة لازالة تلك العناصر الخطرة من مياه الصرف الصناعى المختلفة سواء فى مصدر التلوث نفسه او فى مرفق معالجة ثم اعادة صرفها. من هذا المنطلق ، استهدف هذا البحث إختبار مدى مقاومة سلالة بكتيريا زانثوموناس كامبستريز – المنتجة للسكريات العديدة – لعنصر الكوبلت فى أوساط نموها ، كذلك تم دراسة خصائص عملية إدمصاص الكوبلت بواسطة أنماط مختلفة من مادة الإدمصاص الحيوى ، وقد أوضحت نتائج الدراسة أن هذه السلالة يمكنها النمو فى وجود ١٠٠ ملليجرام كوبلت فى اللتر دون تأثير على إنتاج البوليسكريد فى بيئة النمو تحت نفس الظروف ، بل على العكس فقد ساعدت هذه الظروف على زيادة إنتاجية البوليسكريد المصاحب للخلايا. وقد تبين من قياس تركيز الكوبلت فى الخلايا البكتيرية والبوليمر الحيوى الناتج أن الكوبلت المرتبط وصل إلى ٦٨% من تركيز الكوبلت فى وسط النمو . كذلك أظهرت الخلايا الحية لبكتيريا زانثوموناس كامبستريز قدرة أعلى على إدمصاص الكوبلت بالمقارنة بمثيلتها غير الحية . وقد تأثرت كمية الكوبلت المتجمعة والمرتبطة بالخلايا – سواء الحية أو غير الحية – المختلطة بالسكريات العديدة بكل من درجة حموضة الوسط وتركيز الكوبلت وتركيز ونوع مادة الإدمصاص الحيوى . وقد خلصت الدراسة الحالية إلى أن خليط الخلايا الحية لبكتيريا زانثوموناس كامبستريز والسكريات العديدة – كوسط إدمصاص حيوى – له القدرة على إدمصاص الكوبلت من الوسط المائى الموجودة فيه بكفاءة تصل الى ٨٠ ملليجرام كوبلت لكل جرام من مادة الإدمصاص.