Characterizatin of Cyanide Degrading Bacteria Isolated from Industerial Wastewater

Atta, M.B.[1], Sabet, K.K.[2], Mostafa, H.E.[1] & Amira M. Rizk[1]

[1]Food Science and Technology Dept., Fac. of Agric., Tanta Univ.

[2]Plant Pathology Dept., Fac. Agric., Cairo Univ.

ABSTRACT

Bacterium that utilizes cyanide compounds (e.g. potassium ferrous cyanide, sodium thiocyanate, amygdalin and formamide) as a nitrogen source was isolated from industrial wastewater of electro plating plant. The isolate could tolerate and grow in the presence of sodium cyanide in the growth medium up to 20 mM. Ammonia, formate and / or formamide were found as metabolites of cyanogenic compounds produced by the isolated bacterium. Maltose and sucrose are better than glucose when used as carbon sources by this isolate. Thus, the isolated bacterium could be survived in different wastes containing maltose or sucrose. The results indicated that, the bacterial isolate was identified as *Escherichia coli*, which can be used as a promising tool to biodegrade and detoxificate wastewater and food staff containing cyanogenic compounds as bitter almond. Further study is recommended.

Keywords: Escherichia coli, cyanide, biodegradation, wastewater

INTRODUCTION

Cyanide is widely used in chemical industries in a variety of process such as metal plating, coal gasification, pharmaceuticals, agro-chemical industries, mining and synthesis of organic compounds (Raybuck, 1992, Watanabe *et al*., 1998). Plants also are potential sources of cyanogenic glycosides which liberate HCN after their hydrolysis (Francisco & Pinotti 2000). As a result of these activities large amount of cyanide is released to the aquatic environment (Towill *et al*., 1978). It is estimated that about three billion liter of cyanide derivatives were released to the environment annually through various industries (Fiksel, 1981).

Cyanide has a great toxic effect on various living organisms including humans. Cyanide reduces ferricytochrome oxidase (an iron-containing metalloprotein) to ferrous cytocrome oxidase which transfers the electrons to O_2 (Jeong *et al*., 2005). Although, there are different chemical and physical degradation methods used to reduce and / or eliminate cyanogenic compounds, huge amounts of toxic by- products are released in the aquatic environment (Karavaiko *et al*., 2000).On the other hand, cyanide biodegradation decrease the accumulation rate of toxic compounds (Adjei & Ohta, 1999). Based on this fact, the strategy to eliminate cyanide containing wastes have turned to biodegradation process using microorganisms. Ingvorsen *et al*., (1991) purified and characterized a cyanide hydolyzing enzyme from *Alcanigenes xylosoxidans* sub sp. *denitrificans*. Furthermore, Kang and Kim (1993) isolated a mixed culture of cyanide degrading bacteria including species of *Serratia*, *Klebsiella pneumnoniae*, *Moraxella* and *Pseudomonas*. Also, Ebbs, (2004) demonstrated that there are four general pathways for the biodegradation of cyanide: hydrolytic, oxidative, reductive, and substitution/transfer.

The present work was carried out to isolate and characterize cyanide degrading bacteria from industrial wastewater in Cairo, Egypt. Moreover, to study the capability of this isolate to use different disaccharides (maltose and sucrose) as a carbon source instead of glucose. Metabolic by-products of cyanogenic compounds via bacterial isolate were also investigated

MATERIALS AND METHODS

Materials

Amygdalin, sodium cyanide, potassium ferrous cyanide, sodium thiocyanate and formamide were obtained from Sigma. All the reagents used in this study were of analytical grade.

Methods

Isolation and identification of bacterial isolate

The strain under study was isolated from wastewater samples from oxidation ponds of an industrial plating factory located in Cairo, Egypt.

Plate dilution method was performed to select an isolate which was purified by the streak -plate method (James and Natalies, 1998). Pure bacterial isolates were tested against NaCN using Luria broth medium (LB) supplemented with NaCN solutions at definite amounts. Plates were incubated at 37°C. Daily visual examination was done to determine bacterial growth as indicated by colony formation. The isolated bacteria were identified according to the characteristics described by Sneath, *et al*., (1986), James & Natalies, (1998) using morphological and biochemical tests.

Testing for cyanide resistance

The Minimal inhibitory concentration (MIC) of each strain was determined according to Babu *et al*., (1995). The isolated strains were grown overnight in test tubes contained LB broth medium which contain different concentrations of either NaCN, $K_3[Fe(CN)_6]$, NaSCN or formamide, individually. The tubes were incubated at 37°C in shacking incubator at 200 rpm for 120 hr. Turbidity measurement was used as an indicator for bacterial growth and was determined by the growth amount using spectrophotometer type Smart spec ™3000, USA at a wave length of 450 nm (Sandor *et al*., 2003).

Cyanide biodegradation

Preparation of bacterial cell suspension

The isolated strain was grown in LB medium with 1 mM NaCN for 24 hr and harvested in the exponential growth phase by centrifugation at 4000-5000 rpm for 15 min.

The pellet was washed up twice with 50 mM phosphate buffer (pH 7.4). A suspension of washed cells was resuspended in the same buffer. The bacterial amount in the prepared suspension was adjusted to reach optical density equals to 1 at a wave length of 540 nm (Watanabe *et al*., 1998).

Assay of cyanide biodegradation

Aliquot 0.5 ml of prepared bacterial suspension was inoculated into 50 ml Minimal medium (M9). The medium containing K_2HPO_4 (1.00g), KH_2PO_4 (3.40g), NaCl (0.45g), KCl (0.50g), $MgSO_4.7H_2O$ (0.50g), $FeSO_4.7H_2O$ (0.01g), Glucose (10.00g per liter) and was sterilized by autoclaving at 121°C for 20 min. Then the medium was supplemented with different concentrations of sodium cyanide (0.2, 0.4, 0.6, 0.8, 1, 1.2mM) in 100-ml Erlenmeyer flasks. The flasks were incubated at 30°C with continuous shaking at 200 rpm. Samples were withdrawn to monitor the amount of cell growth via measuring the optical density at a wave length of 540 nm and the ammonia content of the culture medium (Adjei & Ohta, 1999).

Effect of carbon source on cyanide biodegradation

The effect of carbon source on cyanide biodegradation was studied using maltose and sucrose as alternatives to glucose at concentration of 1% in the growth medium as a carbon source on cyanide biodegradation. Turbidity measurements were used as indicator for bacterial growth where it was measured by using a spectrophotometer (Smart spec ™3000, USA) at a wave length of 540 nm according to Adjei & Ohta, (1999), Figuerira *et al*., (1996) and Sandor *et al*. (2003).

Determination of Substrate Specificity

The ability of the isolated strain to utilize cyanide related compounds was checked by growing the bacterial isolate on different organic and inorganic cyanogenic compounds. Washed cells of bacterial isolate were suspended in 50 mM phosphate buffer (pH 7.4) with an $O.D_{540}$ of equal 1. Aliquot 0.5ml of the suspension was inoculated into 50 ml M9 medium. A substrate NaCN, $K_3[Fe(CN)_6]$, NaSCN or formamide was added to a final concentration of 0.2 % (w/v). The culture was incubated at 30°C and at 200 rpm for 120 hr and the amount of growth was determined as described by Adjei & Ohta, (1999).

Detection of the metabolic by-products of some cyanogenic compounds

The ability of bacterial isolate to degrade various cyanogenic compounds was studied by the determination of the formate and formamide as secondary metabolic by-products (ammonia is main metabolic product) using HPLC analysis with a SCX 10µ (4.6mm × 25cm) Ion-exchange column. The mobile phase was 5 mM H_2SO_4 maintained at a rate of 0.5ml/min. The column was maintained at room temperature.

Absorbance was measured at 210 nm (Adjei & Ohta, 1999). This estimation was occurred out using M9 medium after bacterial growth on each cyanogenic compound.

Determination of cyanide and ammonia in the growth medium

The M9 growth medium was centrifuged at 15,000 rpm for 10 min at 4˚C. An aliquot (50 µl)

of the supernatant was taken to determine cyanide concentration according to Fisher and Brown (1952) by adding 100 µl of 0.5 % (w/v) picric acid in 0.25 M Na_2CO_3 solution. The mixture was boiled for 5 min in a water bath and cooled to room temperature using tap water. The total volume was completed to 1000 µl with about 0.85 ml distilled water. The absorbance was measured at a wave length of 520 nm against a blank of distilled water and picric acid reagent. A standard curve was performed using sodium cyanide solutions (0.05to 0.3mM) according to Adjei & Ohta, 1999.

Ammonia was assayed by mixing 0.5 ml the sample with an equal volume of 1:3 diluted commercial Nessler's reagent. The mixture was mixed well and absorbance of solution was measured spectrophotometericlly at a wave length of 420 nm using ammonium chloride as standard (0.05 to 0.3 mM) according to Adjei & Ohta, (1999).

Statistical analysis

All experiments in this study were analyzed using complete randomized design according to Steel & Torrie (1980).

RESULTS AND DISCUSSION

Identification of the bacterial isolate

The morphological and physiological characteristics of the isolated bacterial strain were summarized in Table (1). These results demonstrated that the isolated strain is suggested to be *Escherichia coli* according to characteristics and properties described by Sneath, *et al*., (1986).

Cyanide resistance of the bacterial isolate

The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the inhibitor above which no growth was observed (Silva-Avalons *et al*., 1990).

The present results indicated that the MIC of the *E. coli* isolate was characterized by high resistance to several cyanogenic compounds such as NaCN, $K_3[Fe(CN)_6]$, NaSCN and amides such as formamide as shown in Table (2) and (3).

Table (2) shows that the highest growth of *E. coli* was detected when the growth medium contained 5 mM NaCN. The growth was decreased as the concentration of NaCN increased. The lowest growth was found when bacterial media contained 20 mM NaCN.

Table 1: Morphological and physiological characteristics of the bacterial isolate

Characteristics	Strain
Morphology	short rod
Mobility	+
Gram's reaction	–
Growth at	
30°C	Grow well
37°C	Grow
Biochemical properties	
Oxidase	–
Catalase	+
Indole	+
Methyl red	+
Voges -Proskauer	–
Citrate	–
Urease	– / F
Sugar fermentation	
Glucose	+ (A)
Lactose	+
Mannitol	+
Resistant to:	
Ampicilin	+
Kanamycin	+
Yellow Pigment	+
Growth in KCN	+

+, positive; -, negative
A, produce acid; F, fermented glucose

Table 2: The effect of NaCN concentration on the growth of *E. coli* isolate

NaCN (mM)	* Amount of bacterial growth
Control	3.694 ± 0.001
5	2.820 ± 0.004^a
10	2.647 ± 0.012^b
15	1.593 ± 0.003^c
20	0.080 ± 0.006^d
25	0.073 ± 0.004^d
26	---
Total mean	1.44
$LSD_{0.05}$	0.01650

In column, means having the same superscript letters are not significantly different
M±SD = Means ± Standard Deviation for three replications (n=3)
* Amount of bacterial growth was expressed as optical density of the growth medium
(LB medium) using spectrophotometer at 540 nm

Table 3. Measurement of Minimal Inhibitory Concentration (MIC) of four cyanogenic compounds on *E. coli* isolate

Cyanide compounds	MIC mM	* Amount of bacterial growth
NaCN	25	0.073
NaSCN	95	0.007
$K_3[Fe(CN)_6]$	75	0.004
Formamide	240	0.090

• Amount of bacterial growth was expressed as optical density of the growth medium.
(LB medium) using spectrophotometer at 540 nm

In conclusion, the MIC value of NaCN for *E. coli* was 25mM. These results could be related to that the *E. coli* isolate tolerated relatively high concentrations of NaCN. Similar results were obtained by Neims & Hellerman, (1962), Skowronski & Strobel, (1969) using *Bacillus pumilius*. Also, in this respect, Adjei & Ohta (1999) stated that strain *Burkholderia cepacia* can tolerate KCN up to 25 mM.

Refering to Table (3) it could be noticed that the resistance of the isolated *E. coli* to NaSCN and formamide was higher than that against NaCN and $K_3[Fe(CN)_6]$. These results were reflected by the growth yield achieved at various substrate concentrations as indicated by MIC of each cyanogenic compound. That is due to the disassociation of NaCN in water yielding toxic CN- ligands. On the other hand, in case of $K_3[Fe(CN)_6]$, cyanide is tightly bound to the K and Fe atoms and thus is unable to impart any toxicity.

Souza- Fagundes *et al*. (2004) mentioned that the MIC of NaSCN for *Pseudomonas* was up to 45 mM which is less than (95mM) of the *E. coli* isolate. That was due to the growth difference of genus and species activity as well.

Cyanide biodegradation

The biodegradation rate was expressed by measuring the bacterial growth rate. Fig. (1) shows that the high growth rate of the isolated *E. coli* is NaCN dependent. As the concentration of cyanide raised in the growth medium, the cell density increased. The growth was gradually decreased up to 0.4 mM by increasing to NaCN concentration.

The growth of *E. coli* was also depended on the type of carbon sources as presented in Table (4). The highest growth rate was observed when the medium contained maltose followed by sucrose and the lowest growth was observed in the case of glucose. Washed cells of *E. coli* isolate failed to grow when NaCN is a sole source of carbon and nitrogen. The growth was observed when sugars were added to the medium as carbon sources.

Metabolic by-products of cyanide

Some bacterial strains are able to degrade cyanogenic compounds producing ammonia as a metabolic substance. The second metabolic by-product is formamide and/or formate depending on the structure of cyanogenic compounds Babu *et al*., (1995).

Table (5) and Fig. (2) show that both of the *E. coli* converted NaCN to ammonia and carbon dioxide in the presence of glucose. On the other hand,

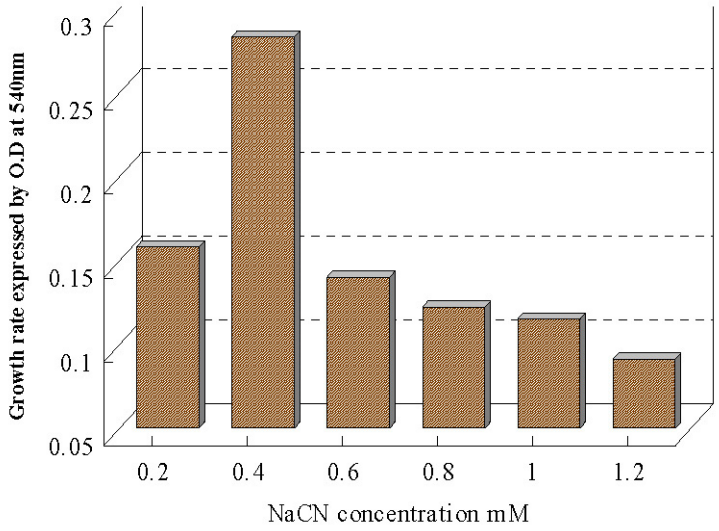

Fig. 1: Effect of NaCN concentration on the growth rate of *E. coli* as indication for the biodegradation process (using M9 medium)

Table 4: Effect of carbon source on the growth amount of the *E. coli* isolate

Carbon Source at a concentration of 1%	* Amount of bacterial growth
Maltose	0.858 ± 0.005[a]
Glucose	0.495 ± 0.004[c]
Sucrose	0.505 ± 0.010[b]

In column means having the same superscript letters are not significantly different at 5% level by DMRT & L.S.D 0.05 = 0.0206

M ± SD = Means ± Standard Deviation for three replications (n=3)

* Amount of bacterial growth was expressed as optical density of the growth medium (using M9 medium containing NaCN) using spectrophotometer at 540 nm

No growth was observed when the glucose was absent from the medium

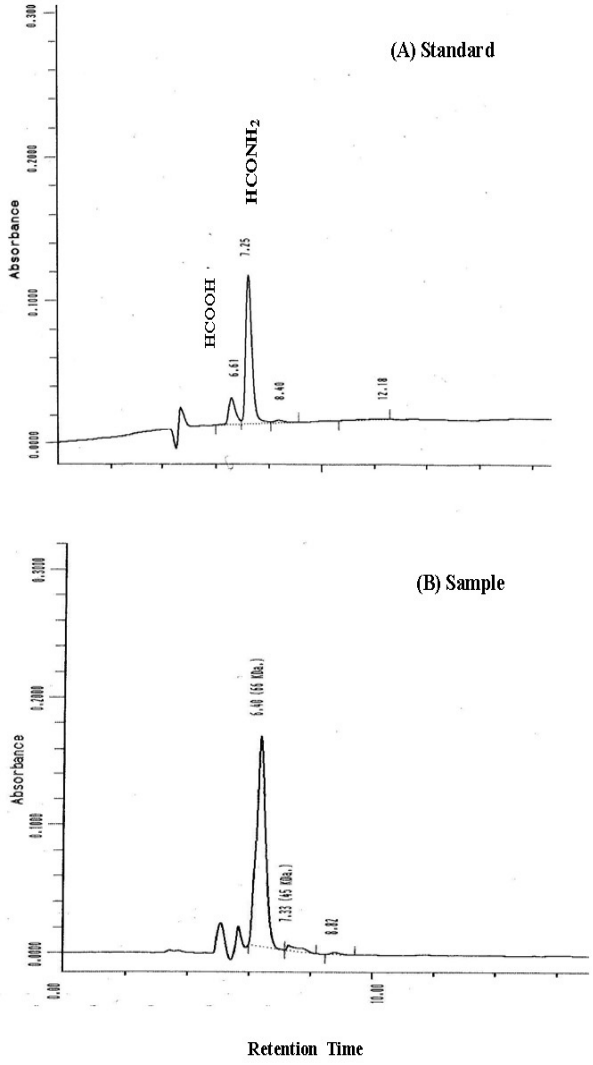

Fig. 2. HPLC chromatogram of metabolic by products of cyanogenic compounds (formate and formamide) after degradation by *E. coli* isolate

Table 5: Metabolic by-products of some cyanogenic compounds after degradation by *E. coli*

Type of Cyanogenic Compound	Metabolic by-products		
	Formamide (mM)	Formate (mM)	NH_3 (mM)
NaCN	----	----	0.980
NaSCN	----	33.500	0.530
K3Fe(CN)6	ND	34.03	0.250
Formamide	----	ND	0.310

---: Not found ND: Found in very low concentratio

the *E. coli* could produced formate as a metabolite for both $K_3[Fe(CN)_6]$ and NaSCN. These results suggest that the bacterial strain had more than one pathway to degrade cyanogenic compound and the end product depended upon the strain type and sort of cyanogenic compounds. These results awere as the trend reported by Faramarzi *et al*., (2004)

The present study demonstrated that the cell suspension prepared from the *E. coli* isolate could convert cyanides, thiocyanates into NH_3 as a final product. The conversion of cyanogenic compounds into NH_3 was catalyzed by the bacterial enzyme (s). Also, the suspension of this isolate is capable to convert formamide into NH_3. This suggests that bacterial enzymes not only convert CN- cyanide group to NH_3 but also convert the amide group of formamide into NH_3.

Substrate specificity

E. coli was capable to grow on a range of cyanide containing substrates as sole source of carbon and nitrogen (Table 6). The previous results dem-

Table 6. Effect of cyanogenic compounds on the growth of isolated *E. coli*

Substrate	Growth amount*
Inorganic	
NaSCN	0.123±0.001
$K_3[Fe(CN)6]$	0.135±0.003
Organic	
Amides (Formamide)	0.123±0.001
CN- glycosides (Amygdaline)	0.510±0.010

Means± Standard Deviation for three replications (n=3)

* Amount of bacterial growth was expressed as optical density of the growth

Medium (using M9 medium containing 0.2% of each substrate) using spectrophotometer at 540 nm

onstrated that the *E. coli* isolate could use organic and inorganic cyanide compounds as sole source of carbon and nitrogen.

In other words, the isolated strain of E. coli, can use inorganic NaSCN and $K_3[Fe(CN)_6]$ as a source of carbon and nitrogen. Further more, it can utilize amygdalin (cyanogenic glycosides) and formamide (amides), as organic cyanide.

In conclusion, the isolated strain of *E. coli* is able to play an important role in degrading cyanide containing compounds which could be found in the environment, or in food stuffs containing cyanogenic compounds. Further intensive investigation is recommended.

REFERANCES

Adjei, D. M. & Ohta, Y. **1999**. Isolation and characterization of a cyanide-utilizing *Burkholderia cepacia* strain C-3. World J Microbiol. Biotechnol., **15**: 699-704.

Babu, V.R. G., Wolfram, H. J., Marian, M. J.& Chapatwala, D. K. **1995**. *Psedomons marginals*: its degrdative capability on organic nitriles and amides. Appl. Microbiol. Biotechnol., **43**: 739- 745

Ebbs, S. **2004**. Biological degradation of cyanide compounds. Current Opinion in Biotechnol., **16** : 231- 235.

Faramarzi, M. A., Stagars, M., Pensini, E., Krebs, W. & Brandl, H. **2004**. Metal solubilization from metal-containing solid materials by cyanogenic Chromobacterium violaceum. J. Biotechnol., **113**: 321-6.

Figuerira, M.M., Ciminelli, S.T.V., de Andrade, C.M. & Linqrdi, R.V. **1996**. Cyanide degradation by an *Escherichia coli* strain. Can. J. Microbiol., **42**: 519 – 523.

Fiksel. J. **1981**. Exposure and Risk Assessment for Cyanide. Office of Water. .United state of Environmental Protection Agency EPA-440-4-85-008 (PB85-220572).

Fisher, F. I. & Brown, J. S. **1952**. Colourimetric determination of cyanide stable gas and water. Anal. Chem., **24**:1440-144.

Francisco, A. I. & Pinotti, P. H. M. 2000. Cyanogenic glycosides in plants. Brazillian Arch Biology Technol., 43: 487- 492

Ingvorsen, K., Højer-Pedersen, B. & Godtrfedsen, E.S. **1991**. Novel cyanide-hydrolyzing enzyme from *Alcaligenes xylosoxidans* subsp. *denitrificans*. Appl. Environ. Microbiol., **57**:1783–1789.

James, G.C. & Natalies, S. **1998**. Microbiology a Laboratory Manual. New York.1-500 .

Jeong, Y., Park, B.G. & Chung, J.S. **2005** High performance biofilm process for treating wastewater discharged from coal refining plants containing nitrogen, cyanide and thiocyanate. Water Sci Technol., **52**: 325-334.

Kang, S. M. & Kim, D. J. **1993**. Degradation of cyanide by a bacterial mixture composed of new types of cyanide degrading bacteria. Biotechnol. Lett., **15**: 201-206.

Karavaiko, G.I.., Kondrateva, T.F., Sarari, E.E., Grigoreva, N.V. & Avakian, Z.A. **2000**. Microbial destruction of cyanide and thiocyanate. Mikrobiol., **69**: 209-216.

Neims, A. H. & Hellerman, L. **1962**. Specificityof the D-amino acid oxidase in relation to glycine oxidase activity. J. Biol. Chem., **237**: PC976-PC978.

Raybuck, S.A. **1992**. Microbes and microbial enzymes for cyanide degradation. Biodegradation, **3**: 3-18.

Sándor, E., Fekete, E. & Karaffa, L. **2003**. Regulation of the Cyanide – Resistant Alternative Respiratory Pathway in the Fungus *Acremonium chrysogenum*. Food Technol. Biotechnol., **41**: 43-47.

Skowronski, B. & Strobel, G. A. **1969**. Cyanide resistance and cyanide utilization by a strain of *Bacillus pumilus*. Can. J. Microbiol., **15**: 93–98.

Silva-Avalons, J., Richmond, G.M., Nagappan,O. & Kunz, A.D. **1990**. Degradation of the metal –cyano complex tetracyanonikellate(II) by cyanide utilizing bacterial isolates. Appl.Environ.Microbiol., **65**: 3664-3670.

Sneath, P.H., Mair, N.S. & Shape, M.E. **1986**. Endospor-forming Gram-positive rods and cocci. In Bergey's Manual of Systematic Bacteriol. Holt J.G. (Ed.) Williams & Wilkins. 999-1581.

Souza-Fagundes, M. E., Rosa, H. L., Gomes, C. M. N., Santos, H. M.& Pimentel, F. P. **2004**. Thiocyanate degradation by pure and mixed cultures of microorganisms. Brazilian J. Microbial., **35**:333-336.

Steel, R.G. and Torrie, J.H. **1980**. Principles and Procedures of Statistics. 2nd (Ed.). McGraw-Hill Book Co. NY, USA.

Towill, L.E. , Drury, J.S., Whitfield, B.L., Lewis, E.B., Galyan, E.L. & Hammons, A.S. **1978**. Review of the environmental effects of pollutants. V. Cyanide. U.S. Environmental Agencey, Cincinnati,Ohio.

Watanabe, A., Yano, K., Ikebukurro, K.& Karube I. **1998**. Cloning and expression of gene encoding cyanidase from *Pseudomonas stutzeri* AK61. Appl. Microbiol. Biotechnol., **50**: 93-97.

عزل و توصيف بكتيريا محللة للسيانيد عزلت من مياه الصرف الصناعي

محمد بسيم عطا[١]، *كامل كمال ثابت[٢]، حبيب السيد مصطفى[١]، أميرة محمد عبد العال رزق[١]

[١] قسم علوم وتكنولوجيا الأغذية، كلية الزراعة ، جامعة طنطا

[٢] قسم أمراض النبات، كلية الزراعة ، جامعة القاهرة

تم عزل بكتريا محللة لمركبات السيانيد (مثل الاميجدالين، سيانيد الصوديوم، الفورمات، الفورماميد ، ثيوسيانات الصوديوم، حديدو سيانيد البوتاسيوم) والتي تقوم باستخدامه كمصدر للنتروجين و ذلك من مياه مخلفات المصانع لأحدى مصادر الطلاء الكهربي بمدينة القاهرة. وقد أوضحت النتائج أن هذه البكتريا المعزولة لها القدرة على التحمل والنمو في وجود سيانيد الصوديوم فى بيئة النمو حتى تركيز أعلى من ٢٠ ملى مول، ويعتبر كل من الأمونيا والفورمات / الفورماميد كنواتج أيضية لمركبات السيانيد المستخدمة بواسطة هذه البكتريا. كما تم استخدام كل من المالتوز، السكروز وكذلك الجلوكوز كمصادر للكربون في بيئة النمو للبكتريا المعزولة. وقد أظهرت نتائج التوصيف والتعريف لهذه السلالة انها *Escherichia coli* والتي يمكن أن تستخدم كأداة جيدة للتكسير الحيوي و إزالة السمية من البيئة المائية و من الأنسجة الغذائية التي تحتوى على مركبات السيانيد