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OPTIMIZATION OF A CULTURE MEDIUM FOR BIOMASS AND δ-ENDOTOXIN PRODUCTION BY A RECOMBINANT *ESCHERICHIA COLI* STRAIN

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ABSTRACT

A recombinant strain of *Escherichia coli* harboring a plasmid containing the *Bacillus thuringiensis* δ-endotoxin synthesis gene, was tested for its efficacy to synthesize δ-endotoxin, in a complex medium containing sucrose and yeast extract. Also, the recombinant *E. coli* strain was tested for its efficacy against the 2nd instars of *Spodoptera littoralis*. The recombinant strain of *E. coli* showed a good activity against the 2nd instars of *S. littoralis,* the mortality was 70 % after 7 days at room temperature. A high cell biomass (8.8gL^{-1}) and δ-endotoxin concentration (6.8 mg L^{-1}), were obtained by the shake flask culture (100 ml medium/250 ml flask, at 200 rpm), of the recombinant *E. coli* in modified MR medium containing sucrose (20g/L), as carbon source and yeast extract as nitrogen source, in the presence of $CaCO_3$, K_2HPO_4, $MgSO_4$, $FeSO_4$ and $ZnSO_4$ as mineral salts. The best pH values for cell biomass production and endotoxin production were 7.0 and 7.5, respectively. The corresponding figures for the best temperature were 37°C and 30°C, respectively. The use of some byproducts such as blackstrap molasses, corn-steep liquor and cheese whey, as an alternative for carbon and nitrogen sources of medium, were found to enhance the cell growth but showed no effect on endotoxin production.

INTRODUCTION

The genetic manipulation of Cry genes of *B.t.* offers a promising mean for improving the efficacy of *B.t.*-based bioinsecticides products **(Chak and Ellar, 1987 and Baum *et al* 1990)**. The Cry genes are localized on large plasmids, the isolation of these genes; their manipulation in vitro and their expression in different organisms are the most powerful and versatile research strategies in modern biology **(Liu *et al* 2000 and Lee *et al* 2005)**.

Mohammed, (2003) and Gamal *et al* (2003) isolated from Egyptian soils, two strains of *Bacillus thuringiensis,* highly effective against the 2nd instars of the cotton leafworm *Spodoptera littoralis*. They found that, CryI gene of these two strains, is responsible for the insecticidal proteins, of the parasporal crystalline inclusions. They also found that maximum spore formation by these two *B.t.* isolates, was attained when glucose and yeast extract were used in medium as carbon and nitrogen sources.

The present study aimed to investigate optimum nutritional and environmental requirements on cell biomass and δ-endotoxin production, by recombinant *E. coli* strain, effective against the 2nd instars of cotton leafworm *Spodoptera littoralis*

MATERIALS AND METHODS

Recombinant *E. coli*

Recombinant *E.coli*, which contains CryI gene, used in the present investigation, was kindly provided from Genetics Dept., Fac. of Agriculture, Cairo University.

The recombinant strain was constructed by cloning the CryIAc gene, (the gene encoding for the insecticidal cryIAc protein that is toxic to Spodoptera pests), from *Bacillus thuringiensis* into the broad host-range plasmid pUC18, as a vector, con-

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taining a constitutive ampicillin promoter that codes for ampicillin resistance, using pUC18 vector ligation reaction(**Abdallah, 1990).**

Bioassay

Crystalline protein isolation

Purification of insecticidal crystal protein (ICP) from recombinant *E. coli* was done according to **Chak and Ellar, (1987)** as follows: cultures were prepared and sonicated, then the disrupted cell suspension was centrifuged and the pellet, containing the toxin proteins, was resuspended in 5 ml sterile distilled water. The inclusions were suspended on sucrose gradients, harvested, washed three times in distilled water and resuspended in distilled water. Samples were stored at -20°C.

Insect bioassay with δ - endotoxin

Cell extracts (1 ml) containing approximately 425 μg proteinious toxin, were brushed on each side of 4-5 cm diameter castor bean leaves discs. The discs were dried in air and then placed in a glass jar with 10 of 2nd instars *Spodoptera littoralis* larvae. The number of dead larvae was recorded after 2 to 7 days incubation at room temperature **(Chak and Ellar 1987).**

Statistical analysis of the bioassay data

Numbers of living and dead insect larvae were counted in control (C) and toxin (T) treatments. As mortality percentage in control larvae ranged from 5 – 20 %, obtained data were corrected according to the **Abbot's formula, 1925,** then, LC_{50} and LC_{90} values were determined.

Data of LC- values at 5 % confidence limits and slopes of regression lines were represented and interpreted using probit analysis statistical method of **Litchfield and Willcoxon, (1949).**

$$\textit{Corrected mortality \%} = \frac{T - C}{100 - C} \times 100$$

Where, LC_{50} = the lethal concentration that kills 50 % of the tested larvae.
LC_{90} = the lethal concentration that kills 90 % of the tested larvae.

Standard curves

Standard curves were constructed to correlate between optical density and cell dry weight, as well as between optical density and endotoxin produced by recombinant *E. coli* strain.

Yet, serial dilutions of recombinant *E. coli* strain were prepared in LB medium **(Atlas, 1998).** After 36 hr incubation at 37°C, cell dry weight and δ- endotoxin produced by recombinant *E. coli* strain were determined according to **Liu *et al* (2000) and Chak & Ellar, (1987).** Also, optical density for growth was recorded spectrophotometrically at 600 nm, according to **(Liu *et al* 2000),** and δ- endotoxin produced by recombinant *E. coli* strain was recorded spectrophotometrically at 280 nm **(Chak and Ellar 1987).**

Cell dry weight and δ-endotoxin production were plotted on X axis, whereas optical density was plotted on Y axis. Cell dry weight of recombinant *E. coli* strain plotted against the optical density obtained by spectrophotometer at 600 nm, were fed to Costat program for statistical analysis, which gave the following simple correlation **(Fig. 1).**

$$Y = 0.27 + 0.2\,X \;.............. \;(1)$$

Where, Y is the absorbance at (600 nm).
X is the cell dry weight (g / L),

Also, δ - endotoxin concentration, produced by recombinant *E. coli* strain, were fed to Costat program for statistical analysis, which gave the following simple correlation **(Fig. 2),**

$$Y = 0.19 + 0.26\,X \;.........\;...\;(2)$$

Where, Y is the absorbance at (280 nm).
X is the δ-- endotoxin concentration (mg / L).

Selection of suitable medium for *E. coli* growth and δ – endotoxin production

Three types of media being LB medium **(Atlas, 1998)** , M9 medium **(Trushin, 2003)** and MR medium **(Wang and Lee, 1997),** were used in this study in order to select the most suitable medium for securing high production of cell dry weight and δ – endotoxin production.

Effect of carbon and nitrogen sources

Sixteen sources of carbon were used instead of the original carbon source in the selected MR medium. Also thirteen sources of nitrogen were used, for preparing the medium. It was put in consideration that the percentage of carbon and nitrogen in

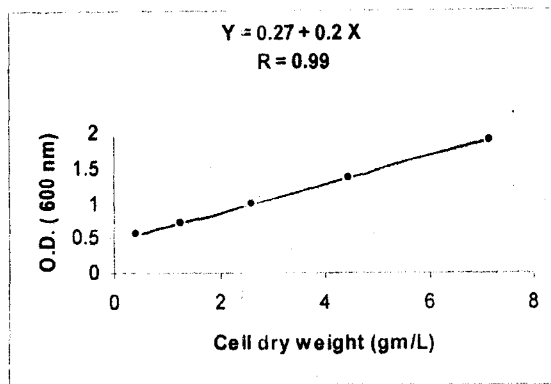

Fig. 1. Standard curve for cell dry weight determination, for recombinant *E. coli* strain

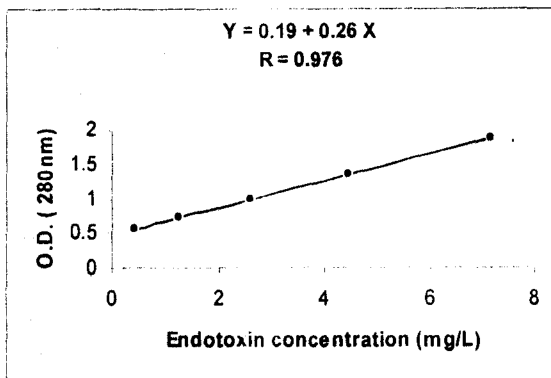

Fig. 2. Standard curve for δ-endotoxin determination produced by recombinant *E. coli*

the alternative sources, were the same with those in the original sources in the selected medium, in order to eliminate errors which may result from the variation of the added sources. The sterile broth medium was inoculated with 1 ml of standard inoculum. Then the inoculated flasks were incubated on a rotary shaker 200 rpm at 37°C for 36 hours. The cell dry weight and δ-endotoxin concentration were determined by spectrophotometer (**Liu *et al* 2000**).

Effect of sugar concentration

Under the best carbon and nitrogen sources, the medium was used with different sugar concentrations (ranging from 10 gm of the carbon source per liter up to 25 gm), to study their effect on cell biomass and toxin production by the tested strain.

Effect of inorganic salts

Five treatments were performed to study the effect of absence of each of KH_2PO_4, $MgSO_4$, $CaCO_3$, $ZnSO_4$ and $FeSO_4$ on cell yield and endotoxin production using the medium, with best tested carbon and nitrogen sources, then the results were compared.

Effect of initial pH

The medium, with best tested carbon and nitrogen sources, was used to study the effect of pH on cell yield and endotoxin production of the recombinant *E. coli*. The media were prepared and adjusted to several different pHs (5.5 up to 9.5) by using standard solutions of HCl and NaOH. The media were sterilized and inoculated with 1 ml of standard inoculum of cell suspension. The cultures were incubated at 37°C for 36 hours. The cell dry weight was determined spectrophotometrically.

Effect of incubation temperature

The modified MR medium was prepared and adjusted to the optimum pH. The sterile medium was inoculated with 1 ml standard cell suspension (0.22 gm dry cells/L) and then incubated at different temperatures (ranging from 20°C to 40°C), then cell dry weight and endotoxin were determined spectrophotometrically.

Effect of aeration

In order to test the effect of aeration on bacterial growth, two procedures were performed by:

1- Using shaking cultures in flasks, containing modified medium, at different speeds ranging from 160–250 rpm, then the cell dry weight and endotoxin concentration at the end of incubation period were determined and compared.
2- Using different volumes of selected medium, being 25, 50, 75, 100 and 125 ml culture medium in the 250 ml shaking flasks, for growing the recombinant *E. coli* strain, with consideration that, the shaking speeds and all the other factors affecting the growth were constant.

Effect of some by-products

Some local by-product materials as alternative carbon and nitrogen sources in MR medium, were used for growth and δ-endotoxin production. These materials were obtained from different sources as follow:

by-product	Source
Black-strap cane molasses	Sugar refinery factory-ElHawamdia
Corn-steep liquor	Glucose and Starch Co. Kozzica
Cheese whey	Egypt dairy products Co. ElAmeria

Molasses (58% carbon), was diluted by addition of water in ratio of 1:1, hydrolyzed at 100°C and pH 5.0 for 60 min, then, pH was adjusted to 7.5 with Ca $(OH)_2$ after cooling to about 60°C, and kept overnight to remove the precipitated undesirable metal salts. The supernatant was sterilized for 30 min at 112°C and added to the medium (14 ml /L) at room temperature.

Cheese whey is the major by-product of cheese manufacturing, representing about 80 % of the volume of milk transformed. It contains approximately 4.5 % (w/v) lactose, which in turn, contains 40% carbon. It was added to the medium in about 400 ml/L.Corn steep liquor contains about 3.5 – 4 % (w/v) nitrogen. It was added to the medium in 16 ml/L.

In preparing the medium, it was put in consideration that the percentage of carbon and nitrogen in the alternative by-product sources, were the same with those in the original sources in MR medium, in order to eliminate errors which may result from the variation of the added sources.

Growth parameters

The modified MR medium was dispensed into 250 ml cotton plugged Erlenmyer flasks, then inoculated with standard inoculum, and incubated for 36 hours on a rotatary shaker. Samples (10 ml) were taken periodically every 3 hours intervals and the cell dry weight and endotoxin concentration were determined spectrophotometrically as mentioned before. The relationship between cell dry weight and time was plotted, specific growth rate and doubling time were calculated from the exponential phase, according to **Painter & Marr (1963),** using the following equation:-

$$\mu = (\ln X_t - \ln X_0) / (t_t - t_0) = \ln 2 / t_d$$

Where,

μ = Specific growth rate (h^{-1})

X_t = Amount of cells at t time (g / liter)

X_0 = Amount of cells at zero time (g / liter)

t_t = time in hours at the end of log phase.

t_0 = time in hours at the beginning of log phase.

t_d = Doubling time (h).

- The δ-endotoxin content (%) was calculated according to **Lee & Chol (1998)** by the following equation:-

gm toxin × 100/ gm biomass dry weight

RESULTS AND DISCUSSION

Toxicity of recombinant *E. coli* against the 2nd instars of *Spodoptera littoralis*

Data in **Table (1) and Fig. (3)** clearly show that, the recombinant strain of *E. coli* revealed a good activity against the 2nd instars of *Spodoptera littoralis*. The recorded mortality was 70 % after 7 days at room temperature. These results are in line with those obtained by **Baum *et al* (1990)** who cloned cry1Ac from *B.t.* subsp. *aizawai* in *E. coli*. They found that, the recombinant strain exhibited good insecticidal activity against *Spodoptera littoralis*.

Selection of suitable medium for production

Escherichia coli is a facultatively anaerobic bacterium, having both a respiratory and a fermentative type of metabolism. Also it is a chemo-organotrophic bacterium that depends on organic compounds as source of carbon and energy, **Sneath *et al* (1986)**.

Data presented in **Table (2)** show the optical density of growth and specific growth rate and δ-endotoxin concentration of recombinant *E .coli* during incubation period in the three tested media (LB, MR and M9 media). Results clearly show that, there was a slight difference in cell biomass between LB and MR media. While there were significant differences in δ-endotoxin concentration using the three types of media. The cell dry weight and the specific growth rate were 8.3 g/L and $0.65h^{-1}$ using LB medium. The corresponding figures for M9 medium were 5.2 gm/L and 0.52 h^{-1}. MR medium exhibited the same trend of LB medium. While, the endotoxin concentration using the three media was 5.1, 5.3 and 2.8 mg/L in LB, MR and M9 media, respectively.

From the aforementioned results, it could be stated that MR medium was preferred for both cell biomass and δ-endotoxin production. So, this medium was chosen for further investigations.

Table 1. Efficacy of recombinant *E. coli* against the 2nd instar larvae of *Spodoptera littoralis*

Bacterial strain	***Cumulative corrected mortality % after indicated days post treatment**					
	2	3	4	5	6	7
Recombinant *E. coli*	0	10	20	40	40	70
B.t. kurstaki **	0	20	30	40	50	80
Control (dist. water)	0	0	0	0	0	0

Standard inoculum suspension contains 0.22 gm dried cells /L.
* Cumulative corrected mortality was calculated according to **Abbott's formula, 1925.**
** Obtained from Cairo MIRCEN – Fac. of Agric. Ain Shams Univ., Cairo.

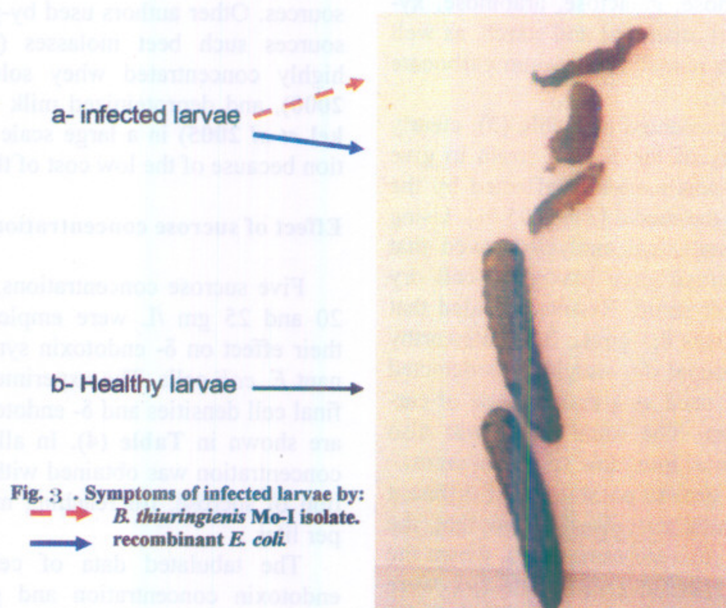

Fig. 3 . Symptoms of infected larvae by:
(red arrow) *B. thiuringiensis* Mo-I isolate.
(blue arrow) recombinant *E. coli*.

Table 2. Cell dry weight, Specific growth rate (μ) and δ- endotoxin concentration of recombinant *E. coli* strain grown on different media using shake flasks as a batch culture, after 36 hours of incubation.

Media type	Cell dry weight (gm/L)			δ- endotoxin		
	O.D.	gm / L	μ (h^{-1})	O.D.	mg / L	Content (%)
LB(1)	1.9	8.32a	0.65	1.5	5.1b	0.060
MR(2)	1.9	8.31a	0.65	1.5	5.3a	0.063
M9(3)	1.3	5.20b	0.52	0.92	2.8c	0.054

- Standard inoculum was 0.22 gm dried cells /L.
- Values in the column having the same letter are not significantly differed, (P = 0.05 %).
- Data were analyzed by **Duncan's multiple range test (1951).**

(1) L.B. (Luria broth) medium (Atlas, 1998), consists of:Yeast extract, 5.00 g; Tryptone, 10.0 g; NaCl, 10.0 g; Dist. water, 1000 ml; pH, 7.00.

(2) MR medium (Wang and Lee, 1997), consists of: $(NH_4)_2HPO_4$,3.0g; KH_2PO_4,2.2g; $MgSO_4.7H_2O$, 0.7g; Trace metal solution, 5ml; Glucose, 20g, Ampicillin (100μg/ ml of medium); Dist. water, 1000 ml; pH,7.0.

(3) M9 medium (Trushin, 2003), consists of:Na_2HPO_4,6g; KH_2PO_4, 3g; NaCl, 0.5g; NH_4Cl,1g; Glucose,10g; Dist. water, 1000 ml; pH,7.5.

Effect of carbon sources

It is quite natural that different carbon compounds vary in their nature and number of carbon atoms. Such various carbon compounds could be used as an energy and carbon sources for bacterial growth. Therefore, it was found of interest to study the influence of the nature of carbon sources on the cell dry weight and δ– endotoxin concentration.

The percentage of carbon present in the MR medium was replaced by equivalent amount of each of the tested carbon source, i.e., organic sources namely, sucrose, glucose, fructose, lactose, maltose, mannose, galactose, arabinose, xylose, ribose, glycerol ,mannitol and starch, as well as inorganic sources namely, potassium carbonate and sodium carbonate.

Tabulated data presented in **Table (3),** clearly show that the ability of the *E. coli* strain to give high yield of cells was markedly affected by the source of carbon in the media [P = 0.05 %]. Using MR medium, the statistical analysis proved that the addition of sucrose gave maximum cell dry weight of the *E. coli* strain. Results revealed that the cell dry weight was 8.5 gm/L. It is noteworthy to state that the minimal dry weight, was detected when mannitol was used as a main source of carbon in MR medium. The same effect was also obtained by addition of inorganic carbon sources.

With respect to growth parameters of different carbon sources, results also clearly show that, the growth parameters gave the same trend, where the highest figures of specific growth rate (μ), were recorded by using sucrose being 0.67 h^{-1} then, by using glucose (0.65 h^{-1}). Similar values of specific growth rates were recorded by using fructose. The lowest values of specific growth rates were observed when using xylose, mannitol, K-citrate and inorganic carbon sources.

In conclusion, it could be stated that, when sucrose was supplemented instead of glucose in the original medium, an obvious improvement of cell yield of the recombinant *E. coli* strain, was achieved, as compared to other tested carbon sources.

Results of endotoxin production indicated that, the highest concentration (6.4 mg/L) and content (0.075 %) was observed on MR medium containing sucrose (20 g/L) as a sole carbon source, after 36 hrs incubation period, followed by glucose, fructose, galactose, mannose and glycerol in descending order. The corresponding figures for endotoxin content (%) were 0.075, 0.061, 0.060, 0.060, 0.064 and 0.07 respectively.

Therefore, sucrose was applied in the following studies. The MR medium, which contains sucrose (20 g/L) instead of glucose, will be designated as MR1.

Comparing the previous data with those obtained by **Liu *et al* (1998),** it was found that, fructose offers a reasonable alternative for glucose in *E. coli* fermentation, since its uptake and utilization are more tightly regulated and higher biomass yields were attained. While, **(Shuishan *et al* 1999 & Liu *et al* 2000 and Marounek *et al* 2003)** preferred glucose as carbon source for growth of recombinant strains of *E. coli* to achieve high cell density in comparison with different carbon sources. Other authors used by-products as carbon sources such beet molasses **(Liu *et al* 1998),** highly concentrated whey solution **(Ahn *et al* 2000),** and deproteinized milk whey powder **(Nikel *et al* 2005)** in a large scale industrial production because of the low cost of these by-products.

Effect of sucrose concentration

Five sucrose concentrations, being 10, 12, 16, 20 and 25 gm /L were employed to investigate their effect on δ- endotoxin synthesis in recombinant *E. coli* cells. The experimental results for the final cell densities and δ- endotoxin concentrations are shown in **Table (4).** In all cases, higher cell concentration was obtained with higher concentration of sucrose, till reaching maximum at 20 gm per liter.

The tabulated data of cell dry weight, δ-endotoxin concentration and growth parameters clearly show that, the highest cell dry weight and specific growth rate (μ) were the highest at 20 gm/L sucrose. The cell dry weight and the specific growth rate were 8.4 gm/L and 0.67 h^{-1} after 36 hr incubation, respectively. Also, endotoxin concentration was higher at the same concentration of sucrose, representing 6.3mg/L with 0.076% content.

Effect of nitrogen sources

This experiment aimed to study the effect of different nitrogen sources on enhancing the growth of the recombinant *E. coli* strain and δ-endotoxin production in MR1 medium. Thirteen nitrogen sources were tested i.e. ten sources of organic nitrogen including amino acids and three inorganic sources. Each source was added instead of ammonium phosphate in amount calculated to give the same concentration of nitrogen in order to

Table 3. Growth parameters of recombinant *E. coli* as influenced by different carbon sources using shake flasks, at 37°C, after 36 h of incubation

Carbon source	Cell dry weight (gm/L)		δ -endotoxin	
	gm / L	μ (h^{-1})	gm / L	Content (%)
Sucrose	8.5^{a}	0.67	6.4^{a}	0.075
Glucose	8.3^{b}	0.65	5.1^{b}	0.061
Fructose	8.2^{c}	0.64	4.9^{c}	0.060
Galactose	6.9^{d}	0.54	4.1^{f}	0.060
Mannose	6.7^{e}	0.58	4.3^{e}	0.064
Lactose	5.9^{j}	0.59	4.0^{g}	0.067
Maltose	6.1^{h}	0.61	4.0^{g}	0.065
Mannitol	4.1^{h}	0.44	2.6^{l}	0.063
Glycerol	6.4^{g}	0.60	4.7^{d}	0.070
Arabinose	6.0^{i}	0.46	3.4^{k}	0.057
Xylose	5.6^{k}	0.54	4.1^{f}	0.066
Ribose	6.0^{i}	0.55	4.1^{f}	0.066
Starch	6.5^{f}	0.50	3.9^{h}	0.060
K-citrate	5.5^{l}	0.50	3.9^{h}	0.065
Na-carbonate	5.4^{m}	0.53	3.5^{j}	0.064
K-carbonate	5.5^{l}	0.50	3.6^{i}	0.065
LSD	0.06		0.10	

- Standard inoculum was 0.22 gm dried cells /L.
- Values in the column having the same letter are not significantly differed.
- Data were analyzed by **Duncan's multiple range test (1951).**

Table 4. Growth parameters of recombinant *E. coli* as influenced by different sugar concentration using shaking flasks, at 37°C, after 36 h of incubation

Sucrose concentration (g/L)	Cell dry weight		δ- endotoxin	
	(gm / L)	μ (h^{-1})	mg / L	Content %
10	7.9^{d}	0.6	4.7^{d}	0.059
12	8.0^{c}	0.63	4.9^{c}	0.060
16	8.3^{b}	0.66	5.3^{b}	0.063
20	8.4^{a}	0.67	6.3^{a}	0.076
25	7.0^{e}	0.55	3.7^{e}	0.050
LSD	0.04		0.30	

- Standard inoculum was 0.22 gm dried cells /L.
- Values in the column having the same letter are not significantly differed.
- Data were analyzed by **Duncan's multiple range test (1951).**

Table 5. Growth parameters of recombinant *E. coli* as influenced by different nitrogen sources using shake flasks, at 37°C, after 36 h of incubation

Nitrogen source	Cell dry weight		δ- endotoxin	
	(gm / L)	$\mu(h^{-1})$	(mg / L)	Content %
Yeast extract	8.8^{a}	0.69	6.7^{a}	0.077
Tryptone	8.6^{b}	0.67	6.0^{b}	0.068
Peptone	8.5^{c}	0.66	6.0^{b}	0.068
Gelatin	7.6^{g}	0.60	4.6^{f}	0.060
Powdered milk	6.0^{j}	0.58	3.4^{k}	0.056
Urea	6.8^{h}	0.64	4.3^{g}	0.063
Sodium glutamate	6.4^{i}	0.62	4.6^{f}	0.070
Serine + Alanine	5.9^{j}	0.57	4.2^{h}	0.070
Alanine + Glycine	5.8^{l}	0.56	4.1^{i}	0.070
Glycine + Serine	5.9^{k}	0.55	4.0^{j}	0.067
$NaNO_3$	8.0^{f}	0.63	4.9^{d}	0.060
$(NH_4)_2HPO_4$ (control)	8.4^{d}	0.67	5.1^{c}	0.060
$(NH_4)_2SO_4$	8.2^{e}	0.65	4.8^{e}	0.059
LSD	0.05		1.1	

- Standard inoculum was 0.22 gm dried cells /L.
- Values in the column having the same letter are not significantly differed.
- Data were analyzed by **Duncan's multiple range test (1951)**.

Table 6. Effect of some industrial by-products on cell dry weight and δ-endotoxin production by recombinant *E. coli* on MR medium, after 36 h at 37°C using shake flasks

By-product	Cell dry weight (gm/L)			δ- endotoxin		
	O.D.	gm / L	$\mu(h^{-1})$	O.D.	mg / L	Content (%)
Black-strap molasses	2.0	9.3^{a}	0.7	1.28	4.2^{c}	0.045
Cheese whey	1.85	7.9^{b}	0.64	1.46	4.9^{a}	0.062
Corn-steep liquor	1.67	7.0^{c}	0.55	1.33	4.4^{b}	0.063

- Standard inoculum was 0.22 gm dried cells /L.
- Values in the column having the same letter are not significantly differed, ($P = 0.05$ %)
- Data were analyzed by **Duncan's multiple range test (1951)**.

Table 7. Effect of inorganic salts on cell dry weight and δ-endotoxin production of recombinant *E. coli* on MR2 medium, after 36 h at 37°C using shake flasks.

Media used	Cell dry weight			δ-endotoxin concentration		
	O.D.	gm / L	%	O.D.	gm / L	Content %
Medium - KH_2PO_4	1.91	8.2^{d}	93	1.75	6.0^{d}	0.073
Medium - $MgSO_4$	1.97	8.6^{c}	98	1.85	6.4^{c}	0.074
Medium – $CaCO_3$	1.87	8.0^{c}	91	1.72	5.9^{e}	0.073
Medium - Fe SO_4	1.98	8.7^{b}	99	1.90	6.6^{b}	0.075
Medium - $ZnSO_4$	1.98	8.7^{b}	99	1.90	6.6^{b}	0.075
(control)	1.99	8.8^{a}	100	1.93	6.7^{a}	0.077
LSD		0.01			0.07	

- Standard inoculum was 0.22 gm dried cells /L.
- Values in column having the same letter are not significantly differed.
- Data were analyzed by **Duncan's multiple range tests (1951)**.

treatment (which contains all tested inorganic salts). The percentage of cell dry weight decrease (in medium lacking any tested salt) ranged between 91 – 99 % for the tested strain. The tabulated data also revealed that the cell dry weight was affected by the absence of each salt. From the aforementioned results, it could be noticed that, the least cell dry weight and endotoxin concentration, was observed when $CaCO_3$ or KH_2PO_4 were omitted from the medium. The corresponding dry weight was 8 and 8.2 gm/L, respectively.

From the previous data of media lacking salts, it is obvious that the most effective inorganic salts in the medium, for mass production, were $CaCO_3$, followed by KH_2PO_4, $MgSO_4$ then $FeSO_4$, which confirm the role of these ions on the growth, as well as confirm their buffering capacity. While the most effective inorganic salts for endotoxin production were KH_2PO_4 followed by $CaCO_3$, $FeSO_4$, $MgSO_4$ then $ZnSO_4$. The absence of Ca^{++}, K^{+}, Fe^{++}, Zn^{++} and Mg^{++} resulted in decreasing the cell dry weight, by 9%, 7%, 1%, 1% and 2%, respectively, the highest cell dry weight was obtained in the control treatment. There were significant differences in cell dry weight and endotoxin concentration between the control and all other treatments.

The importance of inorganic salts, in recombinant *E. coli* growth media, was studied by **Ming and Kaspar, (1998).** They reported that, the addition of NaCl (1%) to the medium, enhances acid tolerance.

Lee *et al* (2005) conducted experiments to compare between *E. coli* MG1655 grown under controled conditions and cells grown with a toxic, sublethal $ZnSO_4$ concentration. Cultures were grown in a defined medium permitting maximum Zn bioavailability. They found that, zinc is an essential trace metal ion for growth, but an excess of Zn is toxic to microorganisms.

Effect of environmental conditions

In the following experiments, MR2 medium was used for studying the effect of environmental factors, namely pH, incubation temperature and aeration on cell dry weight and endotoxin production by recombinant *E. coli* strain. Experiments were done on rotary shakers using shake flasks.

a- Initial pH

Five levels of pH, ranging between 5.5 and 9.5 were chosen for this study. Changes in the density of the cultures, also endotoxin concentrations were monitored during growth, using modified MR2 medium at different pH values. It is well known that the pH of the growth medium is of great importance for cell density.

The medium was inoculated with standard inoculum suspension and incubated at 37°C for 36 hr, and at the end of the experiment the cell dry weight and endotoxin concentration were determined. Data presented in **Table (8)** reveal that there were significant differences in cell dry weight and endotoxin concentration with all tested pH values. The proper pH value which gave high cell density for the tested strain was 7.0(the control), while the highest concentration of endotoxin was obtained when initial pH was adjusted at 7.5. These results indicate that, the pH 7.5 was the most favorable one for endotoxin production (6.8 mg/L) by recombinant *E. coli*. The corresponding figure for content (%) was 0.078 %. All other tested pH values, either lower or higher than 7.5, decreased endotoxin production. The effect of pH on the growth of recombinant *E. coli* was studied by **Hyun *et al* (2001).** They tested the amount and activity of glycosyltransferase, produced by recombinant *E. coli*, as affected by different pH values. They found that, the activity was increased by lowering the culture pH to 5.8 as compared to the enzyme produced at pH 7.0.

b- Incubation temperature

Temperature is one of the most important environmental factors influencing the growth and survival of bacteria. In the following experiment, MR2 medium with optimum pH value was used for studying effect of incubation temperature.

In this respect, six degrees of incubation temperature in addition to the control treatment were used, to study the temperature effect on cell density and endotoxin production by recombinant *E. coli* strain using shake flasks as batch culture. Data presented in **Table (9)** clearly show that the increasing of incubation temperature, from 20°C to 37°C, increased cell dry weight, where the highest dry weight value being (8.8 gm/L) was noticed at 37°C incubation temperature. While the maximum concentration of endotoxin was obtained at 30 °C or 33 °C representing a diverse in the best degree of temperature for cell biomass or endotoxin production. The same conclusion was noticed by **Hyun *et al* (2001)** who stated that, the recombinant enzyme produced by recombinant *E. coli* increased by lowering the culture temperature from 37°C to 30°C. Also, **Huang *et al* (2002)** found that recombinant bacterial pencillin acylase

Table 8. Effect of initial pH on cell dry weight and δ-endotoxin production of recombinant *E.coli* on MR2 medium, after 36 h at 37°C using shake flasks

		Initial pH						
		5.5	6.5	7.5	8.5	9.5	7.0 Control	LSD
Cell dry weight	gm/L	7.9^{e}	8.3^{c}	8.7^{b}	8.0^{d}	7.5^{f}	8.8^{a}	0.07
	%	86	94	99	91	81	100	
Delta-endotoxin	mg/L	6.0^{d}	6.1^{c}	6.8^{a}	6.0^{d}	5.6^{e}	6.7^{b}	0.04
	Content (%)	0.075	0.073	0.078	0.075	0.074	0.077	

- Values in columns having the same letter are not significantly differed.
- Standard inoculum was 0.22 gm /L.
- Data were analyzed by **Duncan's multiple range tests (1951).**

Table 9. Effect of incubation temperature on cell dry weight and δ-endotoxin production of recombinant *E. coli* on MR2 medium, after 36 h using shake flasks.

Temperature (°C)	Cell dry weight		δ-endotoxin concentration	
	gm/L	%	mg/L	Content (%)
20	6.0^{g}	68	3.0^{f}	0.050
25	7.0^{f}	79	3.5^{e}	0.050
28	7.5^{e}	81	4.3^{d}	0.057
30	8.0^{c}	91	6.8^{a}	0.078
33	8.1^{b}	92	6.8^{a}	0.080
37 (control)	8.8^{a}	100	6.5^{b}	0.076
40	7.9^{d}	90	5.8^{c}	0.075
LSD	1.3		0.08	

- Values in columns having the same letter are not significantly differed.
- Standard inoculum was 0.22 gm /L.
- Data were analyzed by **Duncan's multiple range tests (1951).**

is usually expressed at low temperatures (less than 30°C), in *E. coli.* Outside this range, cell physiology was seriously affected.

C- Aeration

1- Effect of agitation speed

Results of recombinant *E. coli* and endotoxin production as influenced by different agitation speeds were presented in **Table (10).** This table indicates that, increasing the agitation speed resulted in increasing the cell dry weight and endotoxin concentration till reaching the maximum (8.8 gm/L & 6.8 mg/L, respectively) during 32 hrs of incubation; at 200 rpm. While, at lower or at higher agitation speed the production was drastically affected especially at higher speeds. At 200 rpm, recombinant *E. coli* grew during the incubation period and recorded the highest cell dry weight and endotoxin concentration.

2-Effect of medium volume

Results of recombinant *E. coli* and endotoxin production as affected by different medium volumes were presented in **Table (11).** Results revealed that, increasing the medium volume, in shake flasks, from 25 to 100 ml / 250 ml flask, resulted in increasing both cell dry weight and

Table 10. Effect of agitation speed on cell dry weight and δ-endotoxin production of recombinant *E. coli* on MR2 medium, after 40 h using shake flasks

Time (h)	Agitation speed (rpm)									
	160		180		200 (control)		220		250	
	CDW (gm/L)	Endotoxin conc. (mg/L)	CDW (gm/L)	Endotoxin conc. (mg/L)	CDW (gm/L)	Endotoxin conc. (mg/L)	CDW (gm/L)	Endotoxin conc. (mg/L)	CDW (gm/L)	Endotoxin conc. (mg/L)
Zero	0.22	-	0.22	-	0.22	-	0.22	-	0.22	-
8	0.23	-	0.24	-	0.26	-	0.24	-	-	-
16	0.8	0.25	0.9	0.3	2.1	0.4	0.9	0.28	-	-
24	3.2	1.8	7.1	2.1	8.0	2.3	4.1	2.0	-	-
32	7.9	5.9	8.2	6.2	8.8	6.8	7.7	4.6	-	-
40	7.2	5.9	8.3	6.2	8.6	6.7	7.0	-	-	-

- Standard inoculum was 0.22 gm /L.

Table 11. Effect of medium volume on cell dry weight and δ-endotoxin production of recombinant *E. coli* on MR2 medium, after 40 h using shake flasks

Time (h)	Medium volume in 250 ml shaking flask									
	25		50		75		100		125	
	CDW (gm/L)	Endotoxin conc. (mg/L)	CDW (gm/L)	Endotoxin conc. (mg/L)	CDW (gm/L)	Endotoxin conc. (mg/L)	CDW (gm/L)	Endotoxin conc. (mg/L)	CDW (gm/L)	Endotoxin conc. (mg/L)
Zero	0.22	-	0.22	-	0.22	-	0.22	-	0.22	-
8	0.22	-	0.22	-	0.24	-	0.26	-	-	-
16	0.74	0.2	0.81	0.25	0.99	0.3	1.0	0.4	-	-
24	2.9	1.7	5.8	1.9	7.2	2.1	4.3	2.3	-	-
32	7.3	5.3	7.9	6.0	8.2	6.3	8.8	6.8	-	-
40	6.8	5.3	7.1	6.0	8.3	6.3	8.6	6.7	-	-

- Standard inoculum was 0.22 gm/L.

endotoxin concentration, till reaching the maximum being 8.8 gm/L & 6.8 mg/L, respectively when using 100 ml medium/flask. While using medium volume over 100 ml/flask resulted in disturbance of motion and in culture contamination. Therefore, it could be recommended to use 100 ml of medium per 250 ml flask for both biomass & endotoxin production. **Coleman *et al* (2003)** studied the influence of agitation, inoculum density, pH and strain on the growth parameters of *E. coli*, using brain heart infusion broth medium. Significant effects of agitation and initial population density were identified at 10°C but not at 19 or 37 °C. Strain viability was more apparent at the boundary conditions of growth of low pH and low temperatures.

On the light of the foregoing results, dealing with the effect of some factors on the cell biomass and δ- endotoxin production, by recombinant *E. coli*, it could be stated that the highest cell dry weight (8.8 gm/L), and the highest endotoxin concentration (6.8 mg/L), could be obtained by providing the following conditions in the medium:

1- Sucrose 20 g/L as carbon source.
2- Yeast extract 4g/L as a nitrogen source and growth promoter.
3- $CaCO_3$, K_2HPO_4, $MgSO_4$, $FeSO_4$ and $ZnSO_4$ as mineral salts.
4- Initial pH at 7.0 for cell growth and at 7.5 for endotoxin production.
5- Incubation temperature at 37°C for cell growth and at 30°C for endotoxin production.
6- Hundred ml of medium / 250 ml flasks at 200 rpm agitation speed.

REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. **J. Econ. Entomol. 18: 265 – 267.**

Abdallah, N.A. (1990). The effect of small fragment substitution in the hypervariable region of the gene coding for δ-endotoxin of *Bacillus thuringiensis*. **Bulletin of Faculty of Agric. Cairo Univ., 41(2): 379-389.**

Ahn, W.S.; S.J. Park and S.Y. Lee (2000). Production of poly 3- hydroxybutyrate by fed-batch culture of recombinant *E. coli* with a highly concentrated whey solution. **Appl. Environ. Micrbiol. 66(8): 3624-3627.**

Atlas, R.M. (1998). Handbook of Microbial Media. 2nd Ed. p. 527, Edited by Parks, L. C. CRC Press, London.

Baum, J.A.; D.M. Coyle; M.P. Gilbert; C.S. Jany and B.C. Gawron (1990). Novel cloning vectors for *Bacillus thuringiensis*. **Appl. Environ. Microbiol. 56(11): 3420-3428.**

Chak, K.F. and D.J. Ellar (1987). Cloning and expression in *Escherichia coli* an insecticidal crystal protein gene from *Bacillus thuringiensis*var. Aizawai HD-133. **J. Gen. Microbiol. 133: 2921-2931.**

Coleman, M.E.; M.L. Tamplin; J.G. Phillips and P.S. Marmer (2003). Influence of agitation, inoculum density, pH and strain on the growth parameters of *E. coli* O157:H7. **International J. Food Microbiol., 83(2): 147-160.**

Duncan, D.B. (1951). A significant test for differences between ranked treatments in an analysis of variance. **Va. J. Sci. 2: 171-189.**

Gamal, Rawia, F.; H.K. Bekhiet; Abeer, M. Mohammed and M. El-Sawy (2003). The effectiveness of formulation types of Egyptian *Bacillus thuringiensis* isolates against the 2nd instar of *Spodoptera littoralis*. **Arab Univ. J. Agric. Sci., Ain Shams Univ., Cairo, 11(2): 485-499.**

Huang, S.W.; Y.H. Lin; H.L. Chin; W.C. Wang; B.Y. Kuo and C.P. Chou (2002). Effect of pH on high-temperature production of bacterial penicillin G acylase in *E. coli*. **Biotechnology Progress, 18(3): 668-671.**

Hyun, J.H.; H.N. Soo; K.D. Hyuk and P.Y. Cheol (2001). Effects of environmental factors on expression of glycosyltransferase in recombinant *E. coli*. **J. Microbiol. & Biotechnol., 11(1): 92-96.**

Lee, S.Y. and J.I. Chol (1998). Polyhydroxyalkanoates biodegradable polymer. In: **Manual of Industerial Microbiology and Biotechnology (2nd Ed.) pp. 616-627.** Demain, A.L. and J.E. Davies (eds). ASM Press, Washington, D.C.

Lee, L.; J.A. Barrett and K.R. Poole (2005). Genome wide transcriptional response of chemostat cultured *E. coli* to zinc. **J. Bacteriol., 187(3): 1124-1134.**

Ling, H.; D. Magnus; E.S. Olof and H. Lena (2001). Effect of glycine on the cell yield and growth rate of *E. coli*: Evidence for cell-density-dependent glycine degradation as determined by 13C NMR spectroscopy. **J. Biotechnololgy, 92(3): 237-249.**

Litchfield, J.T. and F. Willcoxon (1949). A simplified method of evaluating dose effect experiments. **J. Pharmacol. Exp. Therap. 96: 99 – 133.**

Liu, F.; W. Li; D. Ridgway and T. Gu (1998). Production of poly-β-hydroxybutyrate on molasses by recombinant *E. coli*. **Biotechnology Letters, 20(4): 345-348.**

Liu, Y.C.; L.C. Liao and W.T. Wu (2000). Cultivation of recombinant *E. coli* to achieve high cell density with a high level of penicillin G acylase activity. **Proc. Natl. Sci. Counc. 24(4): 156-160.**

Marounek, M.; E. Skrivanova and V. Rada (2003). Susceptibility of *E. coli* to C2-C18 fatty acids. **Folia Microbiologica, 48(6): 731-735.**

Ming, C.C. and C.W. Kaspar (1998). Growth and processing conditions affecting acid tolerance in *E. coli* O 157. H7. **Food Microbiology-London. 15(2): 157-166.**

Mohammed, Abeer, M. (2003). Microbiological Studies on *Bacillus thuringiensis* Production. pp. 89-91. M.Sc. thesis, Fac. of Agric., Ain Shams Univ., Cairo.

Nikel, P.I.; M.J. Pettinari; B.S. Mendez and M.A. Galvano (2005). Statistical optimization of a culture medium for biomass and poly β- hydroxybutyrate production by a recombinant *E. coli* strain using agroindusterial byproducts. **International Microbiology 8: 243-250.**

Painter, P.R. and A.G. Marr (1963). Mathematics of microbial populations. **Annual Rev. Microbiol. 22: 229-234.**

Shuishan, S.; H. Silke and S. Alexander (1999). Production of poly β hydroxybutyric acid by fed-batch culture of recombinant strain of *E. coli*. **Biotechnology Letters, 21(3): 193-197.**

Sneath, P.H.A.; N.S. Mair; E.M. Sharpe and J.G. Holt (1986). Bergey's Manual of Systematic Bacteriology. 1st Ed., Vol. 2, Edited by Williams & Wilkins Press, Baltimore, U.S.A.

Trushin, M.V. (2003). Studies on distant regulation of bacterial growth and light emission. **Microbiology, 149: 363-368.**

Wang, F. and S.Y. Lee (1997). Production of poly β-hydroxybutyrate by fed-batch culture of filamentation-suppressed recombinant *E. coli*. **Appl. Environ. Microbiol. 63(12): 4765-4769.**

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الانتاجية المزرعية المثلى للكتلة الحيوية والتوكسين الداخلي، لبكتيريا ايشيريشيا كولاي المحولة وراثيا

[٣]

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المقدمـــــه

تم اجراء هذا البحث بهدف اختبار فعالية سلالة بكتيرية ، ايشيريشيا كولاي، محولة وراثيا لتحتوي على الجين CryI ، المسئول عن تكوين توكسين بروتيني منقول اليها من بكتيريا *Bacillus thuringiensis* المسببة للأمراض الحشرية ، وقد اختبرت السلالة المحولة وراثيا ضد يرقات العمر الثاني من آفة دودة ورق القطن الكبرى *Spodoptera littoralis* .

كذلك تم تنمية هذه السلالة المحولة وراثيا معمليا في بيئات متخصصة ، بهدف الحصول على أعلى انتاجية لكل من الخلايا والتوكسين البروتيني و ذلك من خلال

أ- البحث عن أفضل مصدر للكربون و النيتروجين.
ب- البحث عن أفضل تركيز للسكر في البيئة.
ج- دراسة تأثير استخدام بعض مخلفات التصنيع الغذائي
د - دراسة تأثير الأملاح المعدنية بالبيئة المستخدمة.
هـ- اختيار أفضل رقم أيدروجيني pH، و أفضل درجة حرارة لكل من التنمية و كذلك لانتاج التوكسين.
و- اختيار أفضل درجة للتهوية من خلال اختيار حجم البيئة الأمثل و كذلك سرعة الرج .

وقد أوضحت النتائج التي تم الحصول عليها

- أن السلالة المحولة وراثيا فعالة ضد يرقات العمر الثاني للآفة (حيث سببت ٧٠ % وفيات، بعد سبعة أيام على درجة حرارة الغرفة، في مجاميع اليرقات المحتبرة).
- وأن أحسن الظروف الغذائية و البيئية لانتاج أعلى تركيز من خلايا السلالة المحولة وراثيا وكذلك التوكسين ، هي باستخدام :-
- السكروز، كمصدر للكربون بتركيز ٢٠ جم للتر، ومستخلص الخميرة كمصدر للنيتروجين ، وذلك باستخدام طريقة المزارع المهتزة (١٠٠ مل بيئة في دورق مخروطي سعة ٢٥٠ مل، و سرعة رج ٢٠٠ rpm)، بينما تباين تأثير درجة الحرارة وتركيز أيون الأيدروجين pH ، فكانت درجة الحرارة المثلى هي ٣٧ ^{o}م و pH ٧ و ذلك بالنسبة لأنتاج الخلايا ، بينما كانت أفضل ظروف بيئية لانتاج التوكسين هي عند درجة حرارة ٣٠^{o}م وpH ٧,٥.
- **كما** أوضحت النتائج أن استخدام بعض مخلفات التصنيع الغذائي مثل المولاس و سائل منقوع الذرة وشرش الجبنة كبديل لمصدر الكربون والنيتروجين في بيئة الانتاج ، قد أدى الى اسراع نمو السلالة المحولة وراثيا ، الا انه لم يؤثر على انتاجية التوكسين.

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