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PRODUCTION OF L-TRYPTOPHAN BY MUTANTS OF *CORYNEBACTERIUM GLUTAMICUM*

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ABSTRACT

L-Tryptophan, is one of the amino acids, also is one of the 9 essential amino acids of human which cannot be synthesized internally by him, microbial production is the main method for obtaining tryptophan for commercial uses. Typical microorganisms used for tryptophan production are *E. coli* and *C. glutamicum*. In this study, random mutations is performed on two strains of C. *glutamicum* to increase tryptophan production, the produced tryptophan measured by bioassay using tryptophan auxotroph *E. coli* (JW1254-2) strain, the mutations are performed using UV radiation. To detect and compare the difference between the parent strains and the muted strains genetically two molecular markers were used start codon targeted polymorphism (SCoT) and Inter-simple sequence repeats (ISSR). Tryptophan remarkably increased after *C. glutamicum* mutation to reach 278.4 µg/ml (1257.4% increment) from one mutated strain. Molecular genetics methods showed an ability to distinguish between the two original strains and their mutants and it showed the importance of the mutation by UV radiation for increasing the productivity of the bacteria to tryptophan.

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INTRODUCTION

Cornebacterium glutamicum is a small, non-motile gram-positive commensal bacterium. It has a rod-shaped morphology with a swelled end like a club. It does not form spores. It has catalase and uses fermentative metabolism to break down carbohydrates. Its discovery was in 1950's in Japan, also it has specific importance in biotechnology because it is nonpathogenic, grows rapidly, has fairly few growth needs, has no extracellular protease secretion, and has a comparatively stable genome **(Mateos et al 2006).**

L Tryptophan which discovered by Hopkins in 1901, is one of the 9 essential amino acids of humans which cannot be synthesized internally by him, and need to be given with nutrition **(Lionella, et al 2016).** Many methods have been recognized till know. These methods could be classified into three categories: chemical synthesis, fermentation and enzymatic methods **(Laszlo et al 2005).** Tryptophan can be produced in enzymatic reactions by two ways. The enzymatic manufacture of L tryptophan from precursors includes a single reaction step, or by a variety of microorganisms with these enzyme activities **(Marzieh, Jamshid and Seyedeh, 2010).**

Typical microorganisms used for tryptophan production are *E. coli*, *B. subtilis,* and *C. glutamicum.* Tryptophan producing strains of these bacteria have been built by the classical mutagenic procedure or recombinant DNA technology **(Lothar**

and Michael, 2005). The strains used in production are mainly classified into the followings three types: Auxotrophic mutants, Regulatory mutants and Genetically modified strains **(Ikeda, 2003).**

Mutagens could be physical, chemical or biological in origin. They may act directly on the DNA, causing direct injury to the DNA, and mainly lead to replication error. Some mutagens affect the chromosomal partition and the replication mechanism. **(Kim and Guengerich, 2005**).

Molecular genetic markers could be classified into two classes: 1) molecular markers that recognize variation at the DNA level e.g.; nucleotide changes, deletion, duplication, inversion and or insertion, 2) biochemical markers that distinguish variation at the gene product level e.g.; changes in proteins and amino acids. The main protein marker is alloenzyme **(White, Adams and Neale 2007).**

Inter-simple sequence repeats (ISSRs) are sites in the genome lined by microsatellite sequences. PCR amplification of these regions by using a single primer produce multiple amplification products that could be used as a dominant multi locus marker system in knowing the genetic variation in different organisms **(Ng and Tan, 2015).** The ISSR are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Each band corresponds to a DNA sequence demarcated by two inverted microsatellites **(Ahmed, 2012).**

Increasing the production of tryptophan is a main target so mutation using UV radiation was performed to achieve this target.

Start codon targeted polymorphism (SCoT), is a simple, new and gene targeted DNA marker depend on the short-conserved region in organism genes **(Zhang et al 2016)**. Primers for SCoT marker study were planned from the conserved region surrounding the translation initiation codon ATG **(Lenka et al 2017)**. SCoT markers are considered as more precise than RAPD and ISSR, and it is proposed that primer length and annealing temperature are not the only factors responsible for reproducibility **(Gorji et al 2011)**. This procedure includes formation of single primers from the short-conserved region adjoining the ATG start codon without knowing any information about genomic sequence. **(Zhang et al 2016)**.

MATERIALS AND METHODS

Bacterial strains

Corynebacterium glutamicum strains

Two wild type strains of ***C. glutamicum*** obtained from Ain shams Center of Genetic Engineering and Biotechnology (ACGEB), Fac. of Agric., Ain Shams University.

E. Coli strains

E. coli auxotroph strain for tryptophan (JW1254-2) was obtained from Coli Genetic Stock Center (CGSC) in Yale University according to **Baba et al (2006)**

Media and solutions

Urea agar media

Was prepared for propagation of *C. glutamicum* according to hand book of media as follows.

Agar	15g
Urea	10g
$Na_2HPO_4 \cdot 12H_2O$	9g
NaCl	5g
KH_2PO_4	1.5g
Meat extract	1g
Yeast extract	1g
$MgSO_4 \cdot 7H_2O$	0.2g
$MnCl_2 \cdot 4H_2O$	20g
$CaCl_2$	1.2g
Glucose solution	100ml (5g glucose/100ml)
DW. up to	1000ml

Minimal salt media (MSM)

MSM was prepared according to **Ahmed et al (2013)** for selection of mutated bacterial cells and for estimation of produced tryptophan amount.

KH_2PO_4	1g
K_2HPO_4	0.6g
$(NH)_2SO_4$	1g
$MgSO_4.7H_2O$	0.5g
DW. up to	1000ml

Maintenance media for *C. glutamicum*

Was prepared according to **Ahmed et al (2013)** for activation of the bacteria.

Meat extract	10g
Peptone	10g
NaCl	5g
DW. up to	1000ml

Luria-Bertani (LB) media

Was prepared for propagation of *E. coli* according to **Luria and Burrous (1955)**.

Tryptone	10g
NaCl	10g
Yeast extract	5g
DW. up to	1000ml

DSMZ Media (451) for *E-coli*

Was prepared according to **Ronald, (2010)** for making the standard curve of tryptophan production.

10X M9 salts	100ml
Glucose solution	10ml
Calcium chloride solution	10ml
Magnesium sulfate solution	10ml
Thiamine solution	1ml
Proline solution	1ml
Tryptophan solution	1ml
DW. up to	1000ml

Stock solutions of DSMZ media 451

10X M9 salts

Na_2HPO_4	60g
KH_2PO_4	30g
NH_4Cl	10g
NaCl	5g
$MgSO_4$	1.2g
DW. up to	1000ml

Glucose Solution

Add 2 g glucose to distilled water and bring volume to 10 ml then autoclave at 15 psi.

Thiamine Solution

Add 3.7 gm thiamine-HCl·$2H_2O$ to distilled water and bring volume to 10 ml then Filter sterilize.

Tryptophan Solution

Add 0.2 g tryptophan to distilled water and bring volume to 10ml then Filter sterilize.

Proline Solution

Add 0.2 g proline to distilled water and bring volume to 10ml then Filter sterilize.

Calcium Chloride Solution

Add 0.1 g $CaCl_2$ to distilled water and bring volume to 10 ml then autoclave at 15 psi.

Magnesium Sulfate Solution

Add 1.2 g $MgSO_4$ to distilled water and bring volume to 10ml then autoclave at 15 psi.

Standard curve of bioassay of L-Tryptophan

Activation of ***E. coli*** in L.B media for 24 hrs. at 37ºC, precipitate the cells and wash them using biological saline. Transfer the cells into minimal media of E. coli (DSMZ 451) **(Ronald, 2010),** added to it different concentrations of tryptophan (0, 20, 40, 60, 80 and 100 µmol. /ml) and incubate them for 24hrs. at 37ºC, dilute them up to the 7^{th} dilution and spread dilutions (5^{th} -7^{th}) on minimal media plates with tryptophan concentration 100µmol/ml, and incubate them for 24hrs. at 37ºC then count the grown colonies and plot the count against the concentration. **(Felix, Holger and Christian, 2012).**

Induced mutation by UV radiation

Induction of mutation using UV radiation according to procedures by **Ahmed et al (2013)** for estimation of produced tryptophan cultivate the mutated *C. glutamicum* in minimal media for then, filtrate the media to obtain bacteria free culture containing the produced tryptophan. And activate E. *coli* tester strain and cultivate in 30 ml minimal media (DSMZ Medium 451 without tryptophan) added to it 200 µl of the filtrate and incubate it for 24 hrs. Dilute the activated up to the 9^{th} dilution, then spread (6-9) dilutions on LB media plates and incubate them for 24 hrs., then count the produced colonies and compare them to the standard curve of the bioassay.

Molecular Markers

Bulked DNA extraction was performed using DNeasy Bacteria Mini Kit (Biobasic INC.). For ISSR molecular markers the DNA amplifications were performed in an automated thermal cycle (model Techni 3000G) programmed for one cycle at 94º C for 4 min followed by 45 cycles of 1 min at 94º C, 1 min at 57º C, and 2 min at 72º C. the final extension was at 72º C for 10 min. the used primers and their sequences were recorded in **(Table**

1). For SCoT molecular markers the DNA amplifications were performed in an automated thermal cycle (model Techni 3000G) programmed for one cycle at 94º C for 4 min followed by 45 cycles of 1 min at 94º C, 1 min at 57º C, and 2 min at 72º C. the final extension was at 72º C for 10 min. the used primers and their sequences were recorded in **(Table 1)**. Finally, agarose gel electrophoresis was performed for the products in Pharmacia submarine filled with TBE buffer **(Welliams et al 1990).**

Table 1. List of ISSR and SCOT primers

Name	Primer sequence (5´→ 3´)
14A	CTC TCT CTC TCT CTC TTG
44B	CTC TCT CTC TCT CTC TGC
HB-9	GTG TGT GTG TGT GC
HB-13	GAG GAG GAG C
HB-15	GTG GTG GTG GC
SCoT 1	ACG ACA TGG CGA CCA CGC
SCoT 3	ACG ACA TGG CGA CCC ACA
SCoT 6	CAA TGG CTA CCA CTA CAG
SCoT 10	ACA ATG GCT ACC ACC AGC
SCoT 11	ACA ATG GCT ACC ACT ACC

Table 2. List of Codes of samples

Code	Strain
1	*C. glutamicum* ACGEB1
2	1st M. *C. glutamicum* ACGEB1
3	2nd M. *C. glutamicum* ACGEB1
4	C. *glutamicum* ACGEB2
5	1st M. *C. glutamicum* ACGEB2a
6	2nd M. *C. glutamicum* ACGEB2a
7	1st M. C. *glutamicum* ACGEB2b
8	2nd M. *C. glutamicum* ACGEB2b
9	1st M. *C. glutamicum* ACGEB2c
10	2nd M. *C. glutamicum* ACGEB2c

RESULTS AND DISCUSSION

Induction of Mutation

This work performed on two strains of *C. glutamicum*, (ACGEB1and ACGEB2), each strain subjected to UV Radiation to induce random mutation. The time of radiation was 5, 10, 20 and 40 minutes. In *C. glutamicum* ACGEB1 tryptophan level before mutation was 19.6 µg/ml while in *C. glutamicum* ACGEB2 was 20.48 µg/ml.

After subjecting of *C. glutamicum* ACGEB1 to UV radiation for 5 mins. the produced tryptophan increased from 19.6 to 27.6 µg/ml with increasing 40.8%. While, the desired mutation did not occur when subjected for 10, 20 and 40 mins. to UV radiation. In *C. glutamicum* ACGEB2, the produced tryptophan increased after being subjected for 5 mins. to UV radiation to 21.6 µg/ml., 30.4 µg/ml, after 20 mins. and 21.8 µg/ml after 40 mins. with increasing 5.5%, 48.4% and 6.5% respectively, while the desired mutation did not occur when subjected for 10 mins. to UV radiation.

When the mutated strains were subjected to the 2nd mutation using UV radiation, the produced tryptophan became 29.4 µg/ml with increment 6.5% than the first mutation in C.G. ACGEB1, 48.8 µg/ml with increment 126% than the first mutation in C.G. ACGEB2a, 278.4 µg/ml with increment 816% than the first mutation in C.G. ACGEB2b and 81.6 µg/ml with increment 274.3% in C.G. ACGEB2c as shown in **(Table 3).**

The final tryptophan yields in this work were 29.4 µg/ml (50% increment), 48.8 µg/ml (138.3% increment), 278.4 µg/ml (1257.4% increment) and 81.6 µg/ml (295% increment) from C.G. ACGEB1, C.G. ACGEB2a C.G. ACGEB2b and C.G. ACGEB2c respectively. The tryptophan yield is remarkably increased especially in C.G. ACGEB2b 278.4 µg/ml, then C.G. ACGEB2c 81.6 µg/ml, also the percentage of increment is markedly increased in the same order.

Table 3. Tryptophan level of *C. glutamicum* strains estimated before and after mutation

Strain	Count	Conc. In micromolar	Amount in micro-gram/ml	% inc. from original	% inc. from 1st mutant
1	$188.6*10^7$	98	19.6		
2	$274.3*10^7$	138	27.6	40.8	
3	$293.5*10^7$	147	29.4	50	6.5
4	$198*10^7$	102.4	20.48		
5	$210*10^7$	108	21.6	5.5	
6	$503*10^7$	244	48.8	138.3	126
7	$304*10^7$	152	30.4	48.4	
8	$297*10^8$	1392	278.4	1257.4	816
9	$211.5*10^7$	109	21.8	6.5	
10	$855*10^7$	408	81.6	295.5	274.3

The results of tryptophan level were obtained by bioassay using tryptophan auxotroph *E. coli* strain, the standard curve of this assay is shown in **(Table 4) and (Fig. 1).**

Table 4. E. coli Auxotroph tryptophan results used as biological assay

Conc. of trp in micro-mollar	Count			Average
zero	77	77	88	$81*10^5$
20	334	326	339	$33*10^7$
40	58	67	55	$60*10^7$
60	59	74	69	$67*10^7$
80	81	87	59	$84*10^7$
100	256	270	286	$270*10^7$

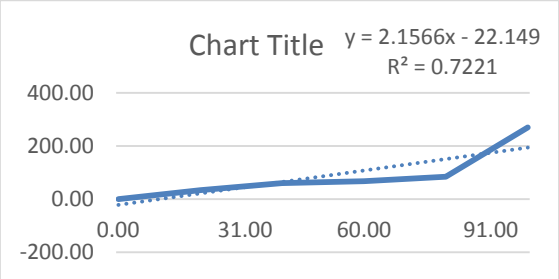

Fig. 1. Standard curve of bioassay of tryptophan

There are many methods for estimating the amino acids, the bioassay used in this work is one of these methods. The bioassay is one of the biosensors, the benefits of using these biologically based assays lie in their specificity, sensitivity and portability and there is no need for large and expensive instrumentation. Biosensors can include enzymes or antibodies, but mainly microorganisms, especially bacteria. Bacteria have the capacity to grow rapidly in relatively inexpensive media. There are many reports on microbial assays for specific amino acids estimation such as tyrosine, tryptophan and glutamic acid (Rf.). *E. coli* is an important bacterium used for bioassay for quantitative and qualitative determination of amino acids including tryptophan. *E. coli* is used mainly because it is characterized by having the lowest doubling time among other bacteria, it is easy to grow and has simple media requirements. *E. coli* genetics is well established and can be easily manipulated to generate mutants with desired characteristics (**Vesela et al 2009**). **Sebek, (1965),** used *Chrombacterium violaceum* to estimate L tryptophan level, and he found that this method is specific for free tryptophan.

In this work, UV radiation which is a physical mutagenic factor used to produce mutation in *C. glutamicum*. The desired mutation was indicated by the growth of bacteria on the culture media in the presence of tryptophan analogue which indicated higher production of tryptophan. The time that the bacteria is exposed to radiation to produce the desired mutation has an important role, when the time is not suitable for each specific strain, no desired mutation occur.

Isamu, Shin and Michiko (1975), using gene mutation on *Brevibacterium flavum* and culturing it on small volume media could produce tryptophan with 500% increase than that collected from the original strain. In the study of **Ikeda et al (1994),** tryptophan production from *C. glutamicum* using

fermentation method, was 50gm/l of tryptophan with 61% increment. In another study of **Ikeda and Katsumata, (1995),** on tryptophan producing strain of *C. glutamicum* they improved the production by 20% using fermentation method. **Roy and Mukhopadhyay** (**2011)** produced L tryptophan from *Aureobacterium flavescens* by mutation with yield of 6.7 gm/l and increment of 56%, while **Tong et al (2012)** produce tryptophan from *E. coli* by mutation reaching 40.2g/l with 11.9% increment. **Alia et al (2015),** used UV radiation with a time of 5, 10, 12, 15 and 20 m. to produce mutation of *Brevibacterium flavum* (old name of *C. glutamicum*) to produce amino acids, and she found that the best time is 10 and 12 min. **Ngwa et al (2007)** also found that the proper time to produce mutation in *B. flavum* with UV is 15m to produce alanine.

In most studies done to increase tryptophan production either on *C. glutamicum* or other bacteria like *E. coli*, fermentation was the used method.

So, there is a minimal information about using small volume of culture media in cultivation *C. glutamicum* as used in this work to produce tryptophan. Also, there is a difference in produced tryptophan amount in our starting bacterial strain before mutation and the bacterial strains they used. So, we depend in comparing with the previous attempts on increased percent not amount of final produced tryptophan. so, in comparison with the results of other studies, the tryptophan yield increasing percent in our study is much higher than the studies.

Molecular Markers

ISSR-PCR polymorphism

PCR amplification of (ISSRs) by using a single primer produce multiple amplification products that we were able to use as a dominant multi locus marker system to screen the genetic variation that took place between the original used strains and the mutated ones. And 4 primers for were used to test 10 samples to figure out the variations that took place.

Primer 14-A

The obtained results of ISSR analysis using 14A are illustrated in (**Fig. 2)** and presented in (**Table 5)** revealed that, the 14A ISSR primer produced 12 DNA fragments with DNA size ranged from 402 to 1496 bp≈.

Table 5. Amplified DNA fragments and their molecular size(bp) produced by primer 14-A in *C. gutamicum* and its mutants

MW	1	2	3	4	5	6	7	8	9	10	Frequency	Polymorphism
1496	-	-	+	+	+	+	+	-	-	-	0.500	Polymorphic
1175	+	-	+	+	+	+	+	+	-	-	0.700	Polymorphic
1031	-	-	-	-	-	-	-	-	+	+	0.200	Polymorphic
994	+	+	-	+	+	+	+	+	-	-	0.700	Polymorphic
813	+	+	+	+	+	+	+	+	-	-	0.800	Polymorphic
682	+	-	+	-	+	+	+	+	+	+	0.800	Polymorphic
631	-	+	-	-	-	-	-	-	+	+	0.300	Polymorphic
552	-	-	-	-	+	-	-	-	-	-	0.100	Unique
514	-	+	-	-	-	-	-	-	-	-	0.100	Unique
474	+	-	-	-	+	+	+	-	+	+	0.600	Polymorphic
460	-	-	+	-	-	-	-	-	-	-	0.100	Unique
402	+	-	-	+	+	+	+	+	-	-	0.600	Polymorphic

HB-13

The PCR products of primer HB-13 produced 8 DNA fragments with size ranged from 506 to 2194 bp≈ ranged from 2 to 6 bands distributed in two types of *C. gluatmicum* and their mutants presented in (**Fig. 2)** and (**Table 6).**

HB-15

The obtained results of ISSR analysis using HB-15 are illustrated in (**Fig. 2)** and presented in (**Table 7)** revealed that, the HB-15 ISSR primer produced 14 DNA fragments with DNA size ranged from 222 to 2591 bp≈.

44-B

The obtained results of ISSR analysis using 44B are illustrated in (**Fig. 2)** and presented in (**Table 8)** revealed that, the 44B ISSR primer produced 11 DNA fragments with DNA size ranged from 229 to 1600 bp≈.

Data in (**Table 9)** showed the ISSR profile of 10 DNA samples representing two types of *C. gluatmicum* and their mutants using ISSR primers. A total of 44 amplified fragments with DNA size ranged from 222 to 2591 bp. the mean number of generated fragments per primer was 11 fragments. The total number of revealed monomorphic DNA fragments was 4 fragments (9%) with an average of 1 monomorphic per primer. No. of unique bands was 11 (25%) bands with an average of 2.75 unique bands per primer and 29 polymorphic without unique, while number of polymorphic with unique was 40 fragments. The level of recorded polymorphism was 90.9%. The results appeared that primer HB-15 and 14-A were highest polymorphic (100% and 100% polymorphism) followed by 44-B primer (81.8% polymorphism), HB-13 primer (75% polymorphism). Primer HB-15 gave the highest total number of bands (14 bands). While, primers 44-B and 14-A gave (11 bands) the lowest total number of bands were 8 bands given by HB-13.

Some specific markers for two types of *C. gluatmicum* were generated by ISSR analysis are listed in (**Table 10)**, 11 markers were scored for the presence of unique bands for bacterial samples, in addition to absence of two specific bands (polymorphic bands). Sample one had one unique bands scored by primers 44-B and also could be distinguished by absence of one band scored by primer HB-13; sample 4 could be distinguished with two specific unique bands by primers HB-15 and 44-B. The two original *C. glutamicum* ACGEB1 and ACGEB2 (samples 1 and 4) have one unique band scored by primer HB-15. Sample 2 had two specific bands scored by primers HB-13 and 14-A; sample 3 could be distinguished with one specific unique band, which generated by primers HB-15; sample 5 could be distinguished with the absence of one polymorphic band which generated by primer 44-B; sample 6 had two specific unique bands generated by primer 44-B; the two samples 5 and 6 (1^{st} and 2^{nd} mutation of C. G. ACGEB2a) unique band generated by prier HB-15; sample 10 could be distinguished with the presence of two specific unique band by primer HB-15 and HB-13; samples 9 and 10 (1^{st} and 2^{nd} mutations of C.G. ACGEB2b) has one unique band generated by primer 14-A. While samples 7,8 and 9 doesn't have any unique bands.

SCoT-PCR polymorphism

Using SCoT targeted DNA markers in our research helped to study the variation occurred in the conserved region surrounding the translation initiation codon ATG in the samples. We used 4 primers in 10 samples to study the variations between original samples and mutated ones to observe the effect of mutation on this area in genome.

SCoT1

The obtained results of SCoT analysis using SCoT1 are illustrated in (**Fig. 2)** and presented in (**Table 11)** revealed that, the SCoT1 primer produced 5 DNA fragments with DNA size ranged from 445 to 847 bp≈.

SCoT10

The obtained results of SCoT analysis using SCoT10 are illustrated in (**Fig. 2)** and presented in (**Table 12)** revealed that, the SCoT10 primer produced 10 DNA fragments with DNA size ranged from 534 to 2276 bp≈.

Scot 11

The obtained results of SCoT analysis using SCoT11 are illustrated in (**Fig. 2)** and presented in (**Table 13)** revealed that, the SCoT11 primer produced 6 DNA fragments with DNA size ranged from 585 to 1199 bp≈.

Table 6. Amplified DNA fragments and their molecular size(bp) produced by primer HB-13 in *C. gutamicum* and its mutants

MW	1	2	3	4	5	6	7	8	9	10	Frequency	Polymorphism
2194	-	-	+	-	+	+	+	+	+	-	0.600	Polymorphic
1740	-	+	-	+	+	+	+	+	+	+	0.800	Polymorphic
1536	-	+	-	-	-	-	-	-	-	-	0.100	Unique
1452	-	-	-	-	-	-	-	-	-	+	0.100	Unique
1223	-	+	+	+	+	+	+	+	+	-	0.800	Polymorphic
901	-	+	+	+	+	+	+	+	+	+	0.900	Polymorphic
703	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
507	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic

Table 7. Amplified DNA fragments and their molecular size(bp) produced by primer HB-15 in *C. gutamicum* and its mutants

MW	1	2	3	4	5	6	7	8	9	10	Frequency	Polymorphism
2591	-	-	-	-	+	+	-	+	-	-	0.300	Polymorphic
157	-	+	+	-	+	+	+	+	+	-	0.700	Polymorphic
1252	+	-	-	+	+	+	+	-	-	-	0.500	Polymorphic
897	-	+	+	+	-	+	+	+	+	+	0.800	Polymorphic
768	+	-	-	+	-	-	-	-	-	-	0.200	Polymorphic
699	-	-	-	-	-	-	-	-	-	+	0.100	Unique
615	-	+	-	-	+	-	+	-	-	+	0.400	Polymorphic
487	-	-	+	-	+	+	+	-	-	-	0.400	Polymorphic
456	-	-	-	-	-	-	-	+	+	+	0.300	Polymorphic
397	-	-	-	+	-	-	-	-	-	-	0.100	Unique
369	-	-	+	-	-	-	-	-	-	-	0.100	Unique
301	-	-	+	-	+	+	+	+	+	+	0.700	Polymorphic
269	-	-	-	-	+	+	+	-	-	-	0.300	Polymorphic
222	-	-	-	-	+	+	-	-	-	-	0.200	Polymorphic

Table 8. Amplified DNA fragments and their molecular size(bp) produced by primer 44-B *C. gutamicum* and its mutants

MW	1	2	3	4	5	6	7	8	9	10	Frequency	Polymorphism
1600	+	+	+	+	-	+	+	+	+	+	0.900	Polymorphic
1304	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
1088	+	-	-	-	-	-	-	-	-	-	0.100	Unique
983	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
857	-	-	-	-	-	+	-	-	-	-	0.100	Unique
767	+	-	+	+	-	+	+	+	+	+	0.800	Polymorphic
634	-	-	-	-	-	-	+	+	+	+	0.400	Polymorphic
630	-	-	+	-	-	-	-	-	-	-	0.100	Unique
536	+	+	+	+	+	-	-	-	-	-	0.500	Polymorphic
412	-	-	-	-	-	+	-	-	-	-	0.100	Unique
229	+	+	-	-	-	+	+	+	+	+	0.700	Polymorphic

Table 9. Total number of DNA fragments, no. of monomorphic, polymorphic, unique bands and polymorphism percentage, generated by tested 4 ISSR primers in *C. glutamicum* and their mutants

	HB-15	44-B	14-A	HB-13	Total
DNA size bp	222-2591	229-1600	402-1496	506-2194	222-2591
No. of mono-morphic	0	2	0	2	4
Mono-morphism %	0	18.18	0	25	9
No. of Unique	3	3	3	2	11
Unique %	21.4	27.27	27.27	25	25
No. of poly-morphic without unique	11	6	8	4	29
No. of poly-morphic with unique	14	9	11	6	40
Total amplified fragments	14	11	11	8	44
Poly-morph-ism %	100	81.8	100	75	90.9

Table 10. *C.glutamicum and its* mutants positive and negative markers obtained from ISSR-PCR and their molecular sizes

	HB-15	44-B	14-A	HB-13
1	-	1088 bp	-	902(-)
2	-	-	515 bp	1536 bp
3	369 bp	-	-	-
4	397 bp	630 bp	-	-
5	-	1600(-)	-	-
6	-	412 bp 857 bp	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	699 bp	-	-	1452 bp

with DNA size ranged from 445 to 847 bp≈.

Table 11. Amplified DNA fragments and their molecular size(bp) produced by primer SCoT1 in *C. gutamicum* and its mutants

MW	1	2	3	4	5	6	7	8	9	10	Frequency	Polymorphism
847	-	-	+	-	+	+	+	-	+	-	0.500	Polymorphic
756	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
584	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
485	-	-	-	-	-	-	-	-	+	+	0.200	Polymorphic
445	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic

Table 12. Amplified DNA fragments and their molecular size(bp) produced by primer SCoT10 in *C. gutamicum* and its mutants

MW	1	2	3	4	5	6	7	8	9	10	Frequency	Polymorphism
2276	-	-	-	-	-	-	-	-	+	-	0.100	Unique
1897	-	-	+	+	+	+	+	+	+	+	0.800	Polymorphic
1703	-	-	-	-	-	+	+	+	+	-	0.400	Polymorphic
1544	-	-	-	-	-	+	+	+	+	-	0.400	Polymorphic
1287	-	+	+	+	+	+	+	+	+	+	0.900	Polymorphic
1072	+	-	+	+	+	+	+	+	+	+	0.900	Polymorphic
910	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
758	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
638	-	+	-	-	+	+	+	+	+	+	0.700	Polymorphic
534	+	-	+	+	+	+	+	+	+	-	0.800	Polymorphic

Table 13. Amplified DNA fragments and their molecular size(bp) produced by primer SCoT11 in *C. gutamicum* and its mutants

MW	1	2	3	4	5	6	7	8	9	10	Frequency	Polymorphism
1199	-	-	+	+	+	+	-	-	-	-	0.400	Polymorphic
1013	-	+	+	+	+	+	+	+	+	+	0.900	Polymorphic
925	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
772	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
652	+	+	+	-	-	-	-	-	+	+	0.500	Polymorphic
585	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic

Scot 6

The obtained results of SCoT analysis using SCoT6 are illustrated in (**Fig. 2)** and presented in (**Table 14)** revealed that, the SCoT6 primer produced 4 DNA fragments with DNA size ranged from 589 to 1143 bp,≈ .

Data in (**Table 15)** showed the SCoT profile of 10 DNA samples representing two types of *C. gluatmicum* and their mutants using SCoT primers. A total of 25 amplified fragments with DNA size ranged from 445 to 276 bp≈. the mean number of generated fragments per primer was 6.25 fragments. The total number of revealed monomorphic DNA fragments was 10 fragments (40%) with an average of 2.5 monomorphic per primer. No. of unique bands was 1 (4%) bands with an average of 0.25 unique bands per primer and 14 polymorphic without unique, while number of polymorphic with unique was 24 fragments. The level of recorded polymorphism was 96%. The results appeared that primer SCoT-10 was the highest polymorphic (80% polymorphism) followed by SCoT-11 and SCoT-6 primers (50% polymorphism), SCoT-1 primer (40% polymorphism). Primer SCoT-10 gave the highest total number of bands (10 bands). While, primer SCoT-6 gave 4 bands the lowest total number of bands.

Some specific markers for two types of *C. gluatmicum* were generated by SCoT analysis are listed in (**Table 16)**, one marker was scored for the presence of unique band for bacteria*l* samples, in addition to absence of three specific bands (polymorphic bands). Sample 1 could be distinguished by absence of two bands scored by primers SCoT-10 and SCoT-11; sample 2 could be distinguished with the absence of one unique band scored by primer SCoT-10; sample 9 has one unique band scored by primer SCoT-10. samples 9 and 10 (1^{st} and 2^{nd} mutations of C.G. ACGEB2b) has one unique band generated by primer SCoT-1. While samples 3, 4, 5, 6, 7, 8 and 10 doesn't have any unique bands.

Table 14. Amplified DNA fragments and their molecular size(bp) produced by primer SCoT6 in *C. gutamicum* and its mutants

MW	1	2	3	4	5	6	7	8	9	10	Frequency	Polymorphism
1143	-	+	-	-	-	-	+	-	-	-	0.200	Polymorphic
888	+	+	+	-	-	+	-	-	-	-	0.400	Polymorphic
786	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
589	+	+	+	+	+	+	+	+	+	+	1.000	Monomorphic

Table 15. Total number of DNA fragments, no. of monomorphic, polymorphic, unique bands and polymorphism percentage, generated by tested 4 SCoT primers in *C. glutamicum* and their mutants.

	SCoT1	SCoT10	SCoT11	SCoT6	Total
DNA size bp	445-847	534-2276	585-1199	589-1143	445-2276
No. of mono-morphic	3	2	3	2	10
Mono-morphism %	60	20	50	50	40
No. of Unique	0	1	0	0	1
Unique %	0	10	0	0	4
No. of poly-morphic without unique	2	7	3	2	14
No. of poly-morphic with unique	2	8	3	2	15
Total amplified fragments	5	10	6	4	25
Poly-morph-ism %	40	80	50	50	60

Table 16. *C.glutamicum and its* mutants positive and negative markers obtained from SCoT-PCR and their molecular sizes

	SCoT1	SCoT10	SCoT11	SCoT6
1	-	1278(-)	1013 (-)	-
2	-	1072 (-)	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	2276 bp	-	-
10	-	-	-	-

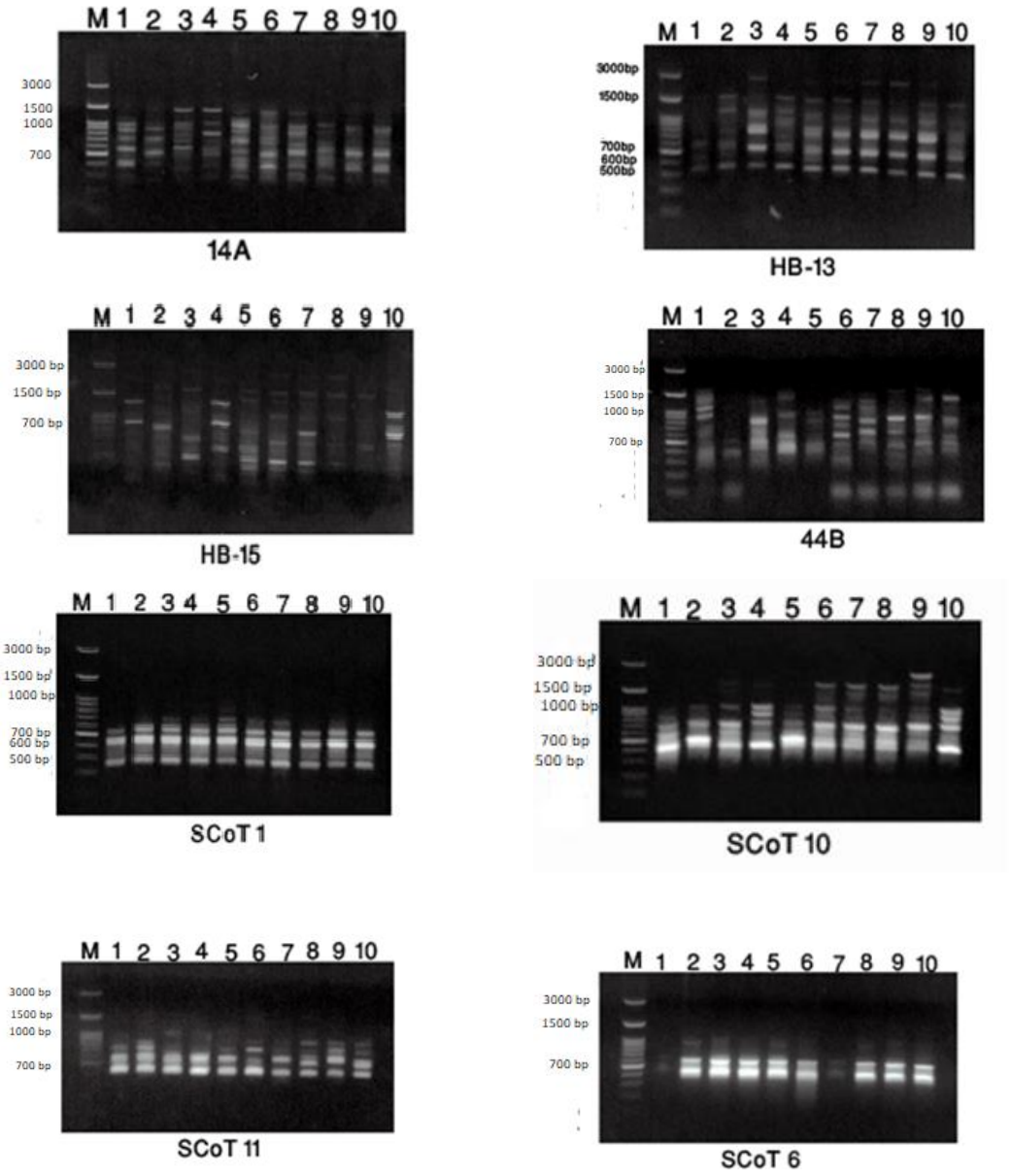

Fig. 2. ISSR and SCoT electrophoretic patterns of *C. glutamicum and its* mutants generated by different primers

CONCLUSION

The final tryptophan yields in this work were 29.4 µg/ml (50% increment), 48.8 µg/ml (138.3% increment), 278.4 µg/ml (1257.4% increment) and 81.6 µg/ml (295% increment) from C.G. ACGEB1, C.G. ACGEB2a C.G. ACGEB2b and C.G. ACGEB2c respectively. The tryptophan yield is remarkably increased especially in C.G. ACGEB2b 278.4 µg/ml, then C.G. ACGEB2c 81.6 µg/ml, also the percentage of increment is markedly increased in the same order. And using different molecular markers showed some significant bands for the mutants produced.

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