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Increased yield of Cyclosporin A obtained using physical and chemical mutagenesis and medium optimization

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Abstract
Cyclosporin A has a history of being recognised as a pharmacologically eminent secondary metabolite. The current study is a concerted attempt to devise a methodology which can lead to higher production of cyclosporin A. Fungus named Tolypocladium inflatum was employed for the production of cyclosporin A. The procured strain was exposed to UV radiations in an intermittent fashion instead of continuous exposure. The selected colonies presenting lowest survival percentage were keenly observed and selected. They were then subjected to chemical mutation using N-nitroso-N-methylurea, at various concentration levels for different incubation time. The effect of mutagenesis on the colonies was analysed and the changes observed were studied in the light of percentage survival and cyclosporin production. Post several rounds of screening and morphological analysis, the stable colonies were eventually used to modify a doubly optimised media. The components of this production media were finalised using Plackett-Burman Design and OVAT study. Mutagenic conditions and medium composition which helped in obtaining maximum cyclosporin production of 5.5 g/L were eventually identified and tested.

Keywords: *Cyclosporin A, Tolypocladium inflatum, UV, N-nitroso-N-methylurea, Plackett-Burman Design, OVAT*

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I. INTRODUCTION

Structurally, the cyclosporins are classified as cyclic peptides comprising 11 amino acids [4]. Cyclosporin A has become the most prominent among the cyclosporins owing to its high specificity as an immunosuppressant [9]. Moreover, it is a clinically approved medicine as a preventative measure against graft rejection in case of organ transplantation [5]. Cyclosporin A also finds profound application in the treatment of autoimmune disorders namely systemic lupus erythematosus, rheumatoid arthritis, psoriasis) [2]. Besides, cyclosporin A has also been studied to retard the growth of parasites, bacteria and fungi [1]. In case of cancer, it plays a significant role in the reversal of resistance against multiple drugs [7]. This remarkable molecule has also been studied in the context of HIV treatment. Naturally, cyclosporin A has gained meticulous attention as an eminent industrial compound [12].

Production of cyclosporin A has been reported from the organisms belonging to fungi or fungi imperfecti, such as *Tolypocladium inflatum* [8], *Aspergillus fumigatis, Aspergillus terreus* [10], *Cylindrocatpum lucidum* [8], *Fusarium roseum, Fusarium oxysporum* [11], *Neocosmospora vasinfecta* [14] and various *Trichiderma* species. But, T. inflatum is the most celebrated microbe when it comes to industrial production of the immunosuppressant because of a comparatively higher productivity. Cyclosporin A has been obtained by various types of fermentations, namely submerged, static and solid state [6]. Enzymatic synthesis has also been observed in this regard. Different types of approaches have also been followed to enhance the yield of cyclosporin and also improvise on its extraction procedure [13]. Redressal of fermentation parameters and conditions applied, optimisation of media, modification of strains to enhance the productivity are some of the commonly followed methodologies in order to obtain secondary metabolites in an increased amount [3].

In the current study, we have made use of *Tolypocladium inflatum* to produce cyclosporin by submerged fermentation. The initial productivity has been recorded then the procured strains have been subjected to intermittent physical mutagenesis, followed by chemical treatment with *N*-nitroso-*N*-methylurea (NMU). Post several rounds of screening, the stable colonies were eventually studied for enhanced productivity. Later, the production media has been optimised to analyse the most significant factors modifying which a better

result could be obtained. Overall outcome of the current study has been promising with regard to a much higher yield of cyclosporin A as compared to the initial production.

II. MATERIALS AND METHOD

2.1 Maintenance of culture

The lyophilized form of T.inflatum was procured from Industrial Lab, Mumbai. It was maintained on plates containing PDA medium, prepared by dissolving 39.0 g/L potato dextrose agar in 1 L of distilled water. Post 10 days incubation at 26 ^{0} C, the serially diluted suspensions of the culture helped in obtaining single colonies. The colonies were keenly analysed for morphology and then selected for performing shake flask activity.

2.2 Shake flask activity

The isolated colonies were studied for their physical attributes and morphology. 2.0 mL of the colony suspension was used to inoculate 50 mL of sterilized lab inoculum medium comprising of corn steep powder (20 g/L), dextrin white (20 g/L), dextrose (10 g/L), KH2PO4 (2 g/L) and MgSO4.6H2O (5 g/L). The flasks were left for incubation on shaking incubator at 26^0 C and 240 rpm for 48 hours. Further, 2.0 mL of the pure mature culture of pH ~5.0 and 10% PMV was transferred to the sterilized lab seed media which was composed of corn steep powder (15 g/L), dextrin white (15 g/L), dextrose (10 g/L), KH2PO4 (2 g/L) and MgSO4.6H2O (5 g/L). Following incubation at 26^0 C and 240 rpm for 44 hours, the mature seed of pH 5.0 and 10% PMV was utilized for transfer to the production media. The production media has the composition as follows:

Table 1: Lab Production Medium

Ingredients	Quantity (g/L)
Corn steep powder	50
Dextrin white	120
Ammonium sulphate	7.5
Urea	2.5
Sucrose	15
pH as such – 5.40 pH Adjusted – 5.80 Sterilization Time – 40 mins at 121°C pH after sterilization – 5.80	

2.3 Quantification methodology

During the course of our entire study, for the purpose of quantification, we have made use of HPLC (Water, pump-alliance (2695), auto sample-alliance (2695); detector –UV (2489) with empower software) method. In order to extract the product from the fermentation broth, methanol has been employed. 5 g of the sample was weighed and dissolved in methanol. The component was then filtered. A mobile phase comprising of 0.01 % orthophosphoric acid and acetonitrile in the ratio of 28:72 was utilised in isocratic mode. For estimating cyclosporin concentration, Waters X-Select-250 x 4.6 mm, 5 µ column was used and the flow rate for the [process was 1.0 mL/min. 20 µL of the resulting solution was injected into HPLC for estimation.

The activity was regularly recorded and analysed and the amount of cyclosporin present in the fermentation broth was determined through the comparison of peak values obtained in the broth to that of the standard.

2.4 Mutagenesis

The initial shake flask experiment was done to understand the productivity scenario of the procured culture colonies. We have later tried to introduce mutagenesis by exposure to UV rays in an intermittent fashion, followed by treatment with chemical mutagen namely *N*-nitroso-*N*-methylurea (NMU), to see if the resultant colonies could be harnessed to produce larger amount of cyclosporin.

2.4.1 Physical mutation

The procured culture was made into a suspension using 5 mL of normal saline solution and then it was serially diluted upto 10-6. 5 mL of the dilutions (10-4, 10-5, 10-6) were distributed directly into sterile petriplates. In a dark room, these plates were exposed to UV radiations of 254 nm, radiating from a distance of 15 cm from the source. This exposure was performed intermittently. The exposure time ranged between 20 to 140 seconds with 3 seconds of no UV rays every 30 seconds. Different plates were allowed to be exposed to UV rays for different intervals of time in order to have a clear picture of the effect of physical mutagen. Then 0.1 mL of the exposed and the unexposed (control) colony suspensions were incubated on PDA at 25^0 C for 10 days.

Post incubation, the samples with around 5% survival rate were subsequently isolated and checked for productivity.

2.4.2 Chemical mutation

The UV mutated spore suspensions were allowed to undergo subsequent screening and the stable colonies were taken for chemical mutagenesis. 1.5 mL of the UV mutated culture suspensions was centrifuged at 5000 g for 10 minutes and the obtained pellet was washed twice in 0.01 M phosphate buffer and the cells were then resuspended in the same buffer. The culture suspensions were separately treated with variable concentration and amount of NMU and incubated for different intervals of time ranging from 1 hour to 12 hours. Post incubations, five serially diluted culture suspensions (10-2 to 10 -6) after NMU treatment, were prepared in normal saline and allowed to grow on PDA for 10 days.

2.4.3 Survival rate determination and the morphological studies

The percentage survival was obtained by determining the ratio of the number of colonies grown after mutagenic treatment to the control. The morphological changes of the colonies for the given doze of mutagen were recorded.

2.5 Medium Optimisation

After studying the effect of mutation on the strains, we wanted to understand the most significant factors which we can modify in order to enhance the productivity of the secondary metabolites. Thus, optimisation of the medium components was done using Plackett-Burman (PB) method and One Variable At a Time (OVAT) model.

PB is oriented towards the identification of the essential components of the medium which could impact the productivity towards a positive result. Here, first-order model has been implicated for the experient which were carried out in a triplicate using equation 1:

Equation 1:

$Y = \beta o + \beta i Xi$ (I = 1,….k)

We have investigated the effect of 5 factors using 2 dummies. The 5 variables, 8 run design was eventually executed and the code sheet for the same has been tabulated below:

Table 2: Code sheet for Plackett Burman design

Independent Factors	Code	Low Value (0) (g/L)	High Value (1) (g/L)
Corn steep powder	X_1	40	80
Dextrin white	X_2	110	220
Ammonium sulphate	X_3	7	12
D_1	X_4	-	+
D_2	X_5	-	+

After PB was performed, OVAT was carried out in order to finalise the concentration of the significant variables. In this experiment, we change the value of one component while keeping the rest of the components at a fixed concentration.

2.6 Final production activity

Shake flask experimentation was finally performed to understand the comprehensive effect of the entire methodological approach followed in the current study.

2.7 Crude extraction

The sample broth which was obtained from the production flask was taken for filtration and then toluene was used to extract the cell mass. The rich solvent was concentrated and mixed with acetonitrile. Then, we had performed carbon treatment and the obtained product was allowed to undergo crystallization. The final product was quantified using HPLC.

III. RESULTS AND DISCUSSION

3.1 Initial production of cyclosporin

This study expresses a comparative result of how much production was achieved when the original culture was allowed to grow on the production medium.

Cyclosporin production using the originally procured strains resulted in the activity recorded as follows:

Table 3: Initial production

Age (Hrs)	pH	PMV (%)	Activity (g/L)
M	5.62	6	.
24	5.20	8	.
48	6.65	10	.
72	6.50	20	.
96	6.31	26	.
120	5.49	28	0.26
144	4.70	34	0.45
168	4.36	38	0.63
192	4.10	40	0.94
216	4.50	40	1.35
240	5.19	38	1.51

Cyclosporin production using the procured strain was found to reach a maximum of 1.51 g/L. Chromatograms of the sample broth have been included below:

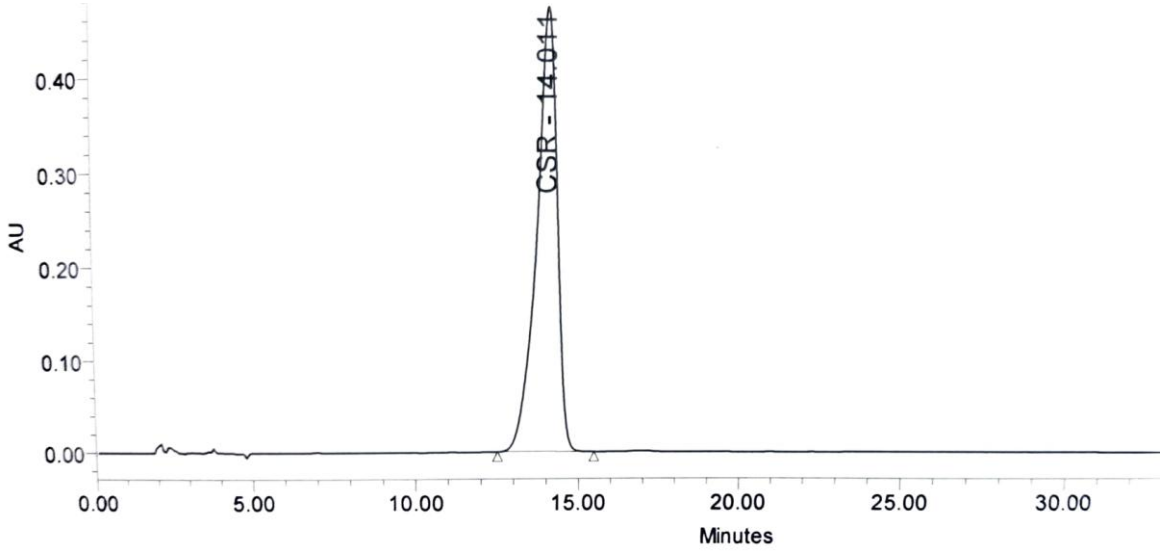

Figure 1: Standard chromatogram for cyclosporin

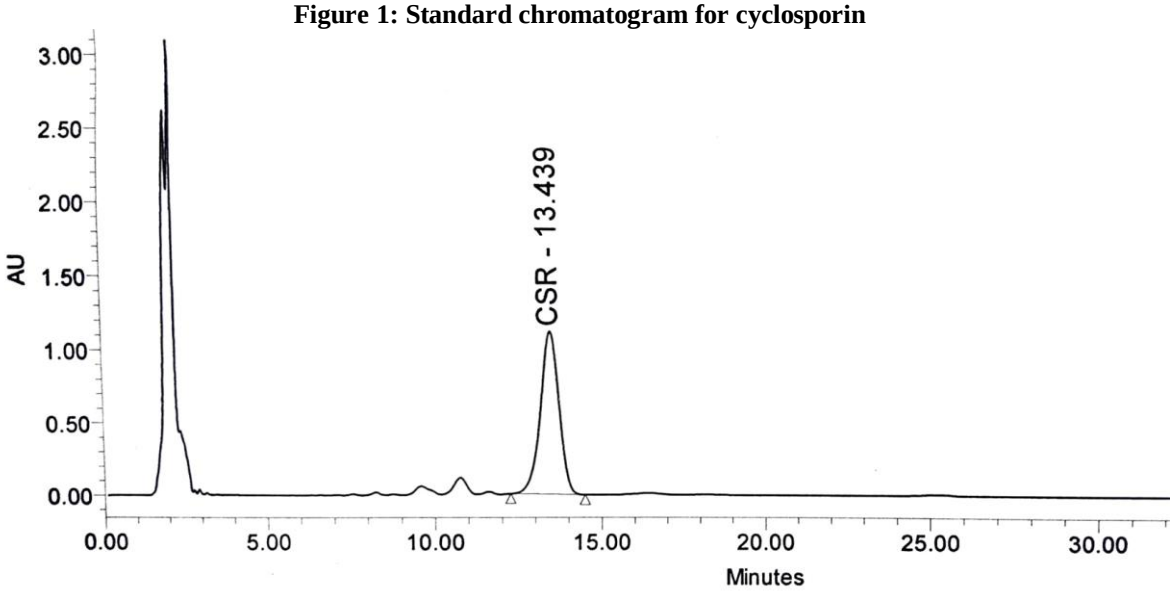

Figure 2: Chromatogram for broth sample

We had tried to introduce mutation in the strain and study if it would impact the colony structure or metabolite production in any way.

3.2 Mutagenesis

On exposing the culture to UV rays, we had tried to study how would an intermittent UV light impact the culture and the morphology of colonies. We found the survival rate to have an inverse proportion with respect to the time of exposure. Precisely, the maximum survival was found to be for the plate exposed for 20 seconds (around 98 %), while for the plates exposed for more than a minute, the survival rate was too less. We did not find much effect of intermittent light, except for that screening of stable colonies was easier once there was a period of no light of 3 seconds between successive exposures. We noticed that post exposure to UV light, the colonies with survival rate of 5.2 %, have shown an enhancement of 0.5 g/L of activity. Thus, the maximum yield obtained was 2.01 g/L of cyclosporin under the effect of UV rays. Further, NMU has been tried on *Tolypocladium inflatum* to study for the survival rate and analyse the effect on the morphology of colonies post treatment.

NMU resulted in certain modifications which could been sighted via microscopic studies. Higher concentration of the mutagen resulted in the death of the colonies. We saw that with the increase in the duration and concentration of the mutagen, decreases the ability to synthesize the secondary metabolite in an appropriate amount.

The survival rates of the colonies has been tabulated to compare the effect of variable NMU concentration for different incubation times:

Table 4: Percentage survival at variable NMU concentration for different incubation times

Incubation time (hours)	Percentage survival (%)		
	For 0.05 % NMU	For 0.07 % NMU	For 0.10 % NMU
2	95	85	75
4	90	73	64
6	86	61	50
8	84	59	34
10	83	55	12
12	78	46	5

The resultant changes in the morphology of the colonies have also been observed as a result of mutation. We found that the colonies prior to any changes appeared round, cotton ball-like but once the dosage of the mutagen (both physical as well as chemical) increased, the peripheral hyphal density kept on decreasing. The images below highlight this difference:

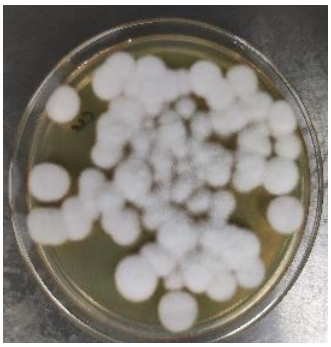

Figure 3: Colony culture prior to mutagenesis

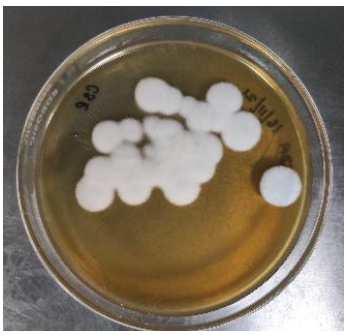

Figure 4: Colony culture obtained after UV exposure

Figure 5: Colony culture obtained after treatment with 0.07 % NMU concentration

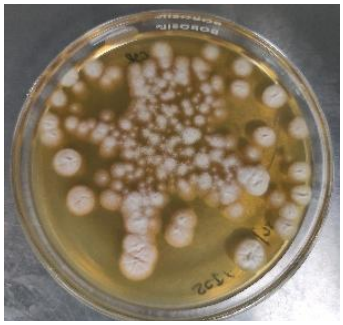

Figure 6: Colony culture after treatment with 0.10 % NMU concentration

3.3 Medium optimisation

3.3.1 Plackett-Burman Design

With the aid of random mutagenesis, we can improvise on the current strains and see the impact on secondary metabolite production. Medium optimisation is another approach by which enhancement in the production activity can be obtained. For our research, we have followed Plackett Burman (PB) method and One-Variable-At-a-Time approach to obtain the precise concentration values of the medium components according to the secondary metabolite being synthesised.

X_1 to X_5 are the major components present in the production media. PB model for 5 factors-2 dummies were screened through 5-variable-8-run design. The design and the response have been tabulated below:

Table 5: Plackett Burman design and its response

Run	X_1	X_2	X_3	X_4	X_5	Response (g/L)
1	1	0	0	1	0	4.00
2	1	1	0	0	1	5.10
3	1	1	1	0	0	4.78
4	0	1	1	1	0	3.90
5	1	0	1	1	1	4.70
6	0	1	0	1	1	3.89
7	0	0	1	0	1	3.10
8	0	0	0	0	0	3.00

The model has brought to notice the significance of corn steep powder and dextrin white. The result pointed out that the 2nd run which conglomerates the higher values for both these components has given the

maximum productivity. This has been further confirmed by the experimental analyses as well. We have then performed OVAT to find the most apt concentration of the significant variables.

3.3.2 One variable at a time method

PB design predicted that corn steep powder and dextrin white impact the productivity to a great extent, thus to make precise evaluation of their values, OVAT was performed. But it was found later that by varying the concentration of corn steep powder, the production recorded increased to a great extent as compared to the changes observed using various amounts of dextrin white. So, OVAT results for corn steep powder have been presented in the table below:

Table 6: Effect of corn steep powder concentration on activity

Concentration (g/L)	Activity (g/L)
20	3.21
40	4.36
60	5.25
80	4.81
100	4.20

Once the precise concentration of the significant variables was found, the production media with the new values was designed and used for the fermentation activity.

Table 7: Final production media

Material	Quantity (g/L)
Corn steep powder	60
Dextrin white	135
Ammonium sulphate	7.5
Urea	2.5
Sucrose	15

3.4 Final production activity

Fermentation was performed using the mutated colonies and the optimised production media and the results have been tabulated below:

Table 8: Final production activity

Age (Hrs)	pH	PMV (%)	Activity (g/L)
M	5.62	6	.
24	5.20	8	.
48	6.65	10	.
72	6.50	20	.
96	6.31	26	.
120	5.49	32	1.95
144	4.70	36	2.89
168	4.36	42	3.21
192	4.10	46	3.85
216	4.50	50	4.43
240	5.19	54	5.50

The maximum concentration of cyclosporin obtained was 5.50 g/L which was quantified by the implication of HPLC.

3.5 Crude extraction results

After 240-hour cycle of fermentation, the production flask broth was filtered to give 157 g of cell mass. This was extracted using toluene. Activity obtained from 10.6 g of extract was found to be 450 mg/g. Finally, 4.98 g of crystallized product gave an activity of 755 mg/g.

Figures 7 and 8 represent the chromatograms of crude sample obtained against the standard.

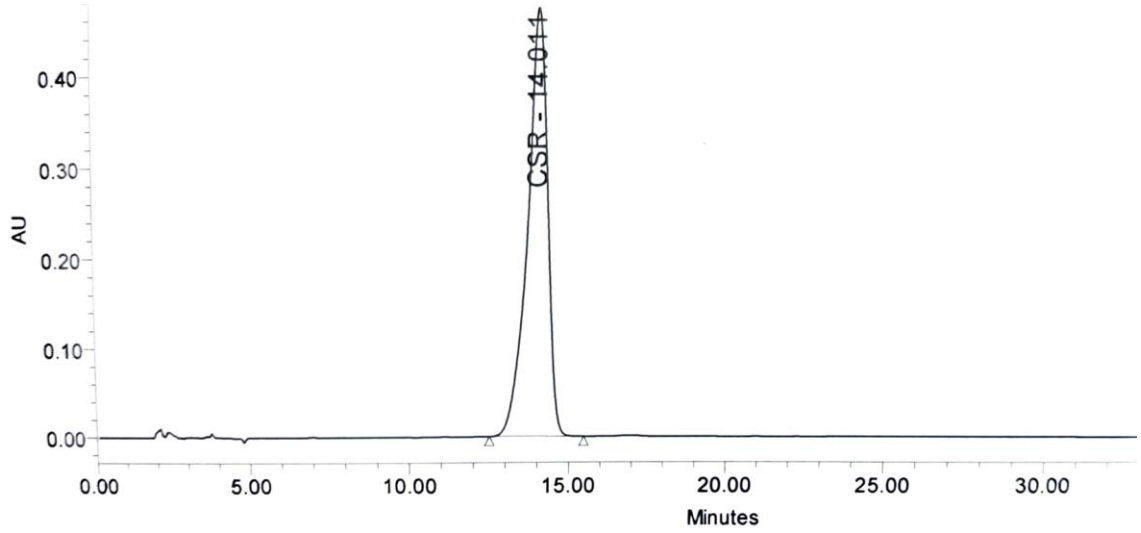

Figure 7: Standard chromatogram for cyclosporin

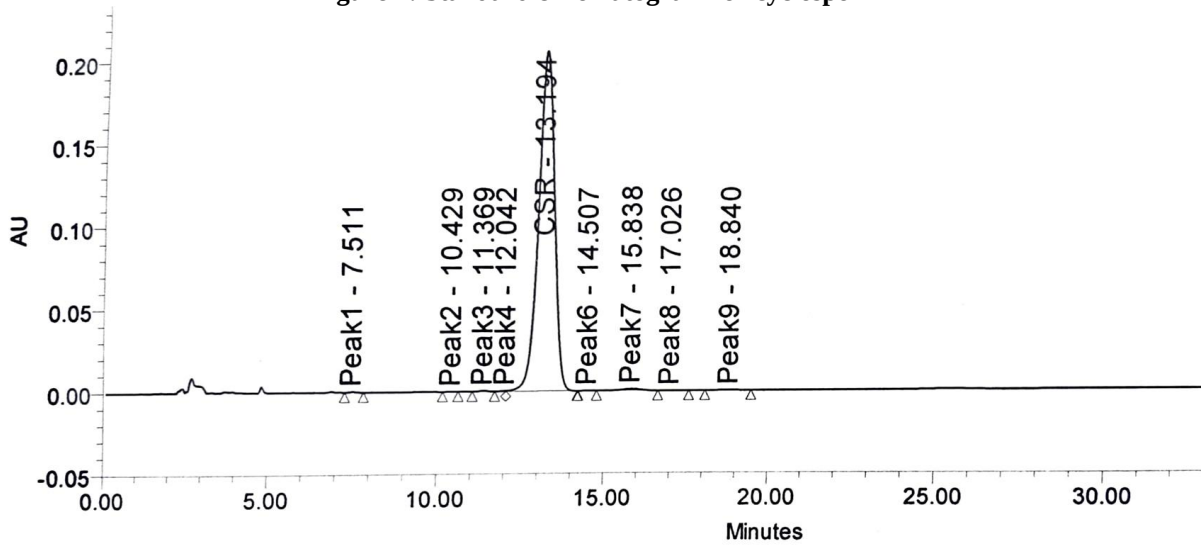

Figure 8: Chromatogram for crude sample

IV. CONCLUSION

On the basis of the current investigation, we have concluded that physical and chemical mutagenesis can be very instrumental in modifying the strains to enhance secondary metabolite production. This study has helped to conclude that the mutagen dose and incubation time are very relevant to the production activity. We obtained colonies which harboured morphological characteristics different from the original strain and consistent for several generations. The colonies resulting from 80 seconds of UV exposure with no light for 3 seconds and 5.2 % survival rate, were studied to be highly stable with respect to morphology. These colonies were selected to study the effect of chemical mutagen, NMU. The most optimum concentration of NMU was found to be 0.07% for 4 hours. In this case, the survival rate was around 73 %. The colonies selected thereby were utilised for shake flask experiment and the medium used was highly optimised. Thus, we were eventually able to obtain the productivity which could be identified by an increase of more than 3.6 times the original value.

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