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Strain Improvement of Mycophenolic Acid Producer Penicillium Brevicompactum Through Mutagenesis

KEYWORDS: *Mycophenolic acid, Penicillium brevicompactum, Ultra violet radiation, Ethyl methane sulphonate, Methyl viologen*

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ABSTRACT *Mycophenolic acid is a immunosuppressive drug, produced by Penicillium brevicompactum, having various other biological properties. Strain improvement of Penicillium brevicompactum was carried out by ultra violet radiation (UV), ethyl methane sulphonate and methyl viologen. The survival percentage of the spores was estimated for different period of exposure of the mutagens. Two mutant colonies were obtained by the mutagenesis experiments. The colonies P120EMS1 and P90MV2 were derived from ethyl methane sulphonate and methyl viologen treatment respectively. The yield varied from mutant to mutant with maximum yield of 10.942mg/g produced by methyl viologen mutant P90MV2 followed by P120EMS1 produced by ethyl methane sulphate mutation giving 10.216 mg/g. Comparative analysis revealed that MV showed an increase in MPA yield by 28 % whereas ethyl methane sulphonate and UV radiation showed an increase of 20 % and 5 % respectively. Additionally, the hereditary stability stud-*

Introduction

Mycophenolic acid (MPA) is a prodrug which is rapidly converted to Mycophenolate mofetil. MPA ($C_{17}H_{20}O6$) is an immunosuppressant which prevents the proliferation of T cells and the formation of antibodies from B cells, thus minimizing the risk of organ transplant rejection (Ransom, 1995). In addition to its immunosuppressive action, it also shows ant ineoplastic,immunosuppressive, antifungal anti-inflammatory, antiviral, and anti-psoriasis activity (Sadhukhan et al, 1991).

MPA is a secondary metabolite, commercially derived from a number of Penicillium species like Penicillium brevicompactum, Penicillium roqueforti, Penicillium scabrum etc through fermentation (Kida et al, 1984). Penicillium brevicomapctum is a ubiquitous soil isolate belonging to the genus Ascomycota. The thallus (mycelium) typically consists of a highly branched network of multinucleate, septate, usually colorless hyphae. Many-branched conidiophores sprout on the mycelia, bearing individually constricted conidiospores. The conidiospores are the main dispersal route of the fungi (Mycology). Extensive research has been carried out for the improved production of MPA which has vast therapeutic impact.

Strain improvement strategies and better production conditions play a very crucial role in enhancing the yield of secondary metabolite in all the fermentation processes (Burg et al, 1979). The microbial strain is the heart of the fermentation industry so, improvement of the strain offers the greatest opportunities for cost reduction without significant capital outlay (Parekh, 2000). Suitable strain improvement techniques can lead to several times greater yield of the metabolite (Demain & Adrio, 2008). The most tangible and widely used tool for strain improvement is mutagenesis (Volff & Altenbuchner, 1998).

The study involves subjecting the strain to physical mutagen like UV radiation and chemical mutagens like Methyl viologen (MV) and Ethyl methane sulphonate (EMS). The selected isolates were tested for MPA production.

Material and Methods

Microorganism and Maintenance of Culture

The ATCC culture of Penicillium brevicompactum was maintained on the Potato Dextrose Agar (PDA) slants. The spores harvested from the slants were used for the mutagenesis experiments.

UV radiation induced Mutagenesis

The UV radiation is the first choice for physical mutagenesis Ultraviolet wavelength is absorbed by pyrimidines, especially thymine. When the energy is absorbed, the ring structure becomes unstable and often leads to the formation of thymine-thymine dimmers. The T:T dimmers do not have normal base pairing properties, so when DNA tries to replicate, the wrong base may be inserted. The thymine thymine dimers form a bulge in the strand leading to hindrance for DNA polymerase in the replication.

Inducing Mutation

The spores were harvested with 5ml normal saline and serially diluted upto $10^{7.}$ 5ml of the last three dilutions(10^5, 10^6,10^7) were distributed into sterile petriplates. The plates were exposed to UV radiation (254 nm) for varying time periods ranging from 5-60 minutes with an interval of 5 minutes in UV chamber keeping the distance of UV source at 40 cm. All these exposures were performed in a dark room to prevent any photoreaction. 0.1 ml of these exposed spores and untreated spores were spread on PDA plates. Plates incubated at 25°C for 12 days. The method employed for the UV mutagenesis was described by Khattab (2012).

Chemical Mutagenesis by EMS and MV

Ethyl methane sulphonate is known to produce random mutations in genetic material by nucleotide substitution, specifically by guanine alkylation. The ethyl group of EMS reacts with guanine in DNA, forming the abnormal base O-6-ethylguanine. During DNA replication, DNA polymerases that catalyze the process frequently place thymine, instead of cytosine opposite O-6-ethylguanine. Following subsequent rounds of replication, the original G:C base pair can become an A:T pair (Irum & Anjum, 2012).

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Methyl viologen is specific to MPA. It exerts a toxic effect on the fungus by inhibiting the enzyme activity catalyzing the synthesis of mevalonic acid from 3 hydroxy-3 methyl glutaryl CoA. Mevalonic acid is an intermediate in the mycophenolic acid synthesis pathway (Kida et al, 1984).

Inducing Mutation

1.5 ml of spores were dispensed in four, 2ml eppendorf tubes. Cells were pelleted and washed twice with 0.01M phosphate buffer after decanting the supernatent. The cells were again resuspended in the same buffer. The cells were split in aliquots of 1ml and 0.5 ml each. the 1ml aliquot was treated with 20 ul of EMS and incubated at 30°C along with 0.5 aliquot tube (Control). The spores were exposed for 0, 30, 60, 90 and 120 minutes. The tubes were processed further accordingly along with control by adding 1 ml of 5% sodium thiosulphate. Again the tubes were centrifuged and the pellet obtained was washed twice with sterile water and once with potato dextrose broth (PDB) and resuspended in PDB. The same procedure was carried out with MV. Each sample was serially diluted to 10^5 and 0.1 ml of dilutions 10^3, 10^4 and 10^5 were spread on PDA plates. Plates were incubated at 25°C for 12 days.

Thereafter, the percentage of survival rate in each plate was calculated using the equation mentioned below, and mutants with the lowest survival rates were selected from the plates (Naveena et al, 2012).

Survival rate = No. of colonies after treatment / No.

of colonies without treatment (M/P) × 100%

Here, C= No. of colonies without treatment (Control)

M = No. of colonies after treatment (Mutant) (Siddique et al, 2014)

The plates with lowest survival rate obtained after exposure to UV, EMS and MV were selected and all the colonies from those plates were picked and screened for MPA productivity.

Stable mutants obtained by chemical and physical were selected based on the consistent expression of the phenotypic character up to 5 generations and maintained on PDA slants for experimental purposes.

Screening of colonies for MPA productivity

The selected colonies were picked and crushed in 5ml normal saline. 1 ml of the spore suspension was inoculated in seed media. Seed media comprises of dextrose 50g//l, soya peptone 15g/l, yeast extract 10 g/l, malt extract 10 g/l, magnesium sulphate 1 g/l, potassium dihydrogen phosphate 1 g/l and sodium nitrate 2.5 g/l, pH 5.8. 1 ml of the harvested spores suspension was inoculated in 35ml seed in 250 ml erlenmeyer flasks. Seed flasks were incubated at 26°C at 240 rpm on shaking incubator for 40 hrs. 8% of the seed was transferred to 250 ml flasks containing 35ml of production media. Basal production media was used for the optimization. Production media composition: sucrose 40 g/l, cotton seed meal 10 g/l, soya flour 25 g/l, casein enzyme hydrolysate 25 g/l, magnesium sulphate 1g/l, potassium dihydrogen phosphate 2.5 g/l, PPG 1 g/l, ammonium sulphate 2 g/l. Flasks were incubated at 26°C and 240 rpm. The yield was assessed through HPLC

HPLC analysis of MPA

Mycophenolic acid produced in the culture broth was determined by HPLC. The culture broth of 2.5 gm was taken in 20 ml volumetric flask with 20 ml methanol and sonicated for 20 minutes and the volume was made up with methanol. The resulting extracted solution was injected into the HPLC (Waters 2496) having C-18 column (Hypersil ODS, 5u C18 (250 mm X 4.6 mm) for the estimation of mycophenolic acid. Concentration of MPA was calculated by comparison of peak areas with those standard mycophenolic acid and subsequently MPA activity was calculated.

Analysis of significant variance among mutants

ANOVA analysis was performed to estimate the variation among the yields of the mutants obtained by the physical and chemical treatment. It was done using online calculator by Daniel Soper (Daniel Soper online calculator version 3.0)

Analysis of hereditary stability

After the screening of the mutant colonies, the condition in which the mutants showed high MPA productivity were optimized and further utilized for checking its stability for five generation. This was carried out for all the mutagens under study i.e. UV radiation, EMS and MV.

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Results and Discussion

One of the promising approach for increasing the production of secondary metabolite by industrially important microorganism is the genetic improvement. The genetic improvement of Penicillium brevicompactum for enhanced MPA production was carried out by physical and chemical mutagenesis. In the present investigation mutation was carried out physically by using UV irradiation and chemically by mutagens like EMS and MV. The dose which allows the lowest survival of fungal colonies was selected for induction of mutation

Selection of UV mutants

The spores of P. brevicompactum were exposed to UV light (254 nm) at a distance of 40cm from the UV source. The survival rate was inversely proportional to the time of exposure of UV (Table1). The least survival rate was 5.71% at exposure time of 60 minutes. The colonies were picked and further tested for MPA production. The yield obtained for the colonies is listed in table 2.

Table 1: Survival rate of UV mutants at different time intervals

Exposure time (minutes)	Test (cfu/ml)	Control (cfu/ml)	% survival
5	24	35	68.57
10	24	39	61.54
15	24	33	72.73
20	20	38	52.63
25	19	36	52.78
30	17	37	45.95
35	15	35	42.86
40	12	34	35.29
45	8	35	22.86
50	5	38	13.16
55	3	32	9.38
60	2	35	5.71

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Table 2: MPA production by UV mutants

Exposure time (mins)	No of colonies	No of MPA producing colonies	Yield (mg/l)
50	5	3	P45uv2- 7.985
			P45uv3-7.458
			P45uv5-5.842
55	3	2	P55uv1-6.741
			P55uv3-7.965
60	2	2	P60uv1-8.209
			P60uv2-8.946

Maximum yield of 8.946mg/gm was seen in mutant P60uv2 obtained at exposure time of 60 minutes. The UV mutagen showed an increase of approximately 5 % in MPA production.

Chemical Mutagenesis

The Penicillium brevicompactum spores were treated with ethyl methane sulphate and methyl viologen. The survival percentage for each treatment at various exposure times is represented in table 3 and 4. The least survival rate was 8.33% for EMS mutants and 3.45% for MV mutants for the exposure time of 120 minutes.The colonies were picked and further tested for MPA production. The yield obtained for the colonies is listed in table 5.

Table 3:Survival rate of EMS mutants

Exposure time (minutes)	Test (cfu/ml)	Control (cfu/ml)	% Survival
0	15	25	60
30	12	22	54.55
60	8	27	29.63
90	5	26	19.23
120	2	24	8.33

Table 4: Survival rate of MV mutants

Exposure time (minutes)	Test (cfu/ml)	Control (cfu/ml)	% Survival
0	14	30	46.67
30	11	27	40.74
60	7	24	29.17
90	3	26	11.54
120	1	29	3.45

Table 5: MPA production by EMS and MV mutants

Mutagen	Exposure time (mins)	No of colonies	No of MPA producing	Yield (mg/l)
EMS	90	5	3	P90EMS1-9.586
				P90EMS3-7.864
				P90EMS4-8.047
	120	2	2	P120EMS1-10.216
				P120EMS2-9.847
MV	90	3	2	P90MV2-10.942
				P90MV3-8.723
	120	1	1	P120MV1-9.753

Maximum yield of 10.216 mg/g was observed in P120EMS1 (EMS mutagen) and 10.942 mg/g in P90MV2 (MV mutagen). EMS mutant obtained at the exposure time of 120 minutes yielded 20 % higher mycophenolic acid than the control strain (8.5 mg/g). MV mutant obtained at an exposure time of 90 minutes yielded 28 % higher mycophenolic acid.

Comparative analysis of MPA production by different mutants with the control strain

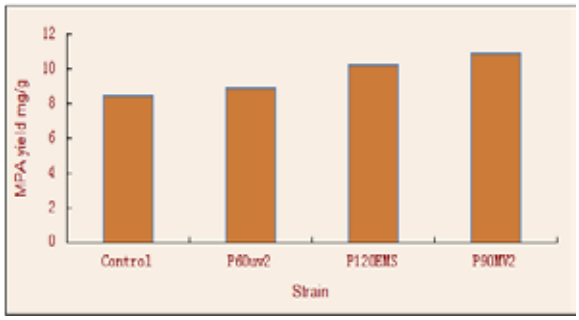

Figure 1: Graphical representation of comparative analysis

From figure 1, it can be observed that methyl viologen mutants have the maximum productivity as compared to UV radiation and ethyl methyl sulphonate. Out of the three treatments employed for the strain improvement, methyl viologen treatment was seen to be the most successful in increasing the MPA productivity.

3.4 Statistical Analysis

ANOVA was conducted to analyze the differences between the mutants in terms of the mutant yield (Table 6.)

Table 6: ANOVA analysis for mutants

Mutant	Mean	SD	F value	p value
UV (P60uv2)	8.946	0.005	16,237.66	0.00
EMS (P120EMS1)	10.216	0.002		
MV (P90MV2	10.942	0.003		
Control	8.5	0.03		

P value less than 0.0500 indicates that the mutant yields varies significantly whereas p values greater than 0.1 indicate that there is no significant variation. In this study it was observed that the different mutants vary significantly from each other as well as from the control.

Phenotypic comparison of the mutants with the parent strain

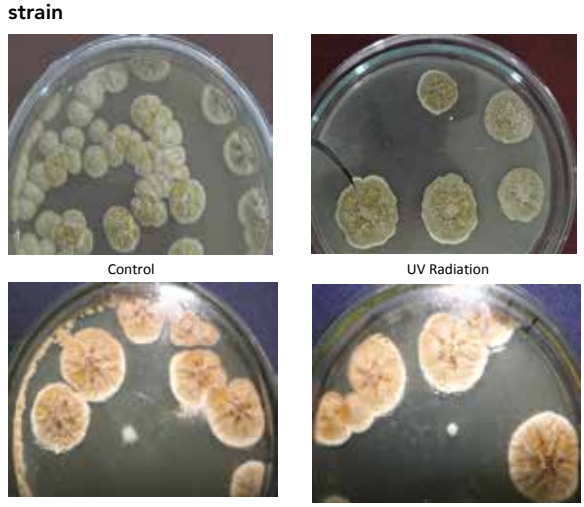

Figure 2: Comparison of mutant strains with parent strain of Penicillium brevicompactum

Visual observation of the plates containing colonies of the parent and the mutant strain was carried out and is depicted in figure 2. It was observed that the colonies were velutinous on the surface with the aerial mycelia emerging out from the centre as spikes. Conidiogenesis ranges from low

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to high as the age increases. It was centrally umbonate. On maturity, furrows appeared on the surface dividing into into sections.

Table 6: Description of mutant and parent Penicillium brevicompactum colonies

Description	Control	UV	EMS	MV
Color	Grey brown	Dark grey	Brown	Brown
Margin	Irregular,round	Irregular,round	Irregular,round	Irregular,round
Size	Small	Medium	Large	Large
Sporulation	+	++	+++	+++

Analysis of Hereditary stability of the screened mutant

Stability studies are very essential to determine the retention of the mutation that enhances the productivity. At times, random mutagenesis can be reverted back in the following generations. The study involved the analysis of the productivity of the mutants over five generations. Since EMS and MV produce high yielding mutants, they were further chosen for this study. The data is represented in table 7. It was observed that the mutants retained the mutation over the generations studied.

Table 7: Stability studies of EMS and MV mutants for 5 generations

Generation	Yield (mg/g)	
	P90MV2	P120EMS1
I	10.942	10.216
II	10.682	10.1
III	10.919	10.31
IV	10.842	9.996
V	10.721	10.153

Conclusion

Strain improvement is one of the most promising area of research which has gained importance in the recent years for enhancing the productivity of secondary metabolite by fermentation. The most conventional method for this purpose is mutagenesis through physical and chemical mutagens. ultra violet radiation (physical mutagen) and ethyl methane sulphonate and methyl viologen (chemical mutagens) were used in the present study to improve the MPA productivity. UV treated mutants gave 5 % increase whereas EMS gave 20 % and MV gave 28 % higher yield than the parent strain. The study also revealed that MV treatment is more promising than the other two. Stability analysis for EMS and MV mutants showed that the mutants were able to retain the mutation and continue to give higher yield through out the various generations. ANOVA analysis of variation showed that the mutants vary significantly among each other in terms of yield.The study also revealed that MV treatment is more promising than the other two. Stability analysis for EMS and MV mutants showed that the mutants were able to retain the mutation and continue to give higher yield through out the various generations.

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