ISSN: 2348-1900
Plant Science Today
http://www.plantsciencetoday.online

Research Article

SPECIAL ISSUE on Current Trends in Plant Science Research

Enhancement of lipase production by ethyl methane sulfonate mutagenesis of soil fungal isolate

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Article history
Received: 27 November 2019
Accepted: 28 December 2019
Published: 31 December 2019

Abstract
Strain improvement through random mutagenesis is an extremely developed practice and it plays an important role in the economic growth of microbial agitation processes. The present study comprises of genetic improvement of fungus isolated from petrol pump soil by ethyl methane sulfonate (EMS) mutagenesis for increased production of extracellular lipase. Random mutagenesis was performed by incubating the spore suspension of fungus with EMS at a concentration of 5% (v/v) and 8% (v/v) for 30, 60 and 90 min, respectively. Control set was prepared by incubating the spore suspension with sterile distilled water. Control plate showed maximum number of fungal colonies whereas number of colonies was decreased as we increased exposure time of EMS from 30 to 90 min. The lipase activity of six mutagenic strains and wild strain was determined under submerged fermentation and solid state fermentation. Treated culture named as $EMS_{5\%-60min}$ (obtained after 60 min exposure with 5% EMS) exhibited maximum activity (32.09 ± 1.84 IU/ml/min) in SmF as compared to wild strain (8.77 ± 3.52 IU/ml/min) and another treated strain named as $EMS_{8\%-90min}$ (obtained after 90 min exposure with 8% EMS) exhibited maximum activity (7.99 ± 0.12 IU/g/min) in SSF as compared to wild strain (1.77 ± 0.71 IU/g/min). The activity of mutagenic strain *i.e.* $EMS_{5\%-60min}$ was increased to 365.90% as compared to 100% activity of wild strain in SmF whereas activity of another mutagenic strain *i.e.* $EMS_{8\%-90min}$ was increased to 451.41% as compared to 100% activity of wild strain in SSF.

Publisher
Horizon e-Publishing Group

Keywords: Ethyl methane sulfonate; fermentation processes; spores; SmF (Submerged fermentation); SSF (Solid State Fermentation); mutagenesis.

Citation: Shreya, Sharma A K, Ritika, Bhati N. Enhancement of lipase production by ethyl methane sulfonate mutagenesis of soil fungal isolate. Plant Science Today 2019;6(sp1):600-606. https://doi.org/10.14719/pst.2019.6.sp1.674

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Introduction

In 1940s the significance of microbial genetics was accomplished during penicillin production. Mutagenesis came in existence by enlargement in productivity by thousand folds at industrial level and also useful for fermentation industry. In commercial growth of microbial agitation the mutagenesis plays a fundamental role. Presently, strain improvement through mutation and assortment is a much developed practice (1). Wild strains obtained by primary and secondary screening produce low quantity of enzymes due to regulatory mechanism which controls the amount of enzyme synthesized; hence wild strains are not suitable for industrial enzyme production (2). The productivity of wild strains can be increased by

Table 1. Lipase production in SmF from fungal isolated strains of petrol pump soil

Fungal isolates	Lipase activity (IU/ml/min)	Protein content (mg/ml)	Specific activity (U/mg)
Strain 1	1.59 ± 0.09	14.54	0.10 ± 0.006
Strain 2	11.98 ± 0.46	14.04	0.85 ± 0.063
Strain 3	0.28 ± 0.05	14.05	0.02 ± 0.002
Strain 4	2.98 ± 0.08	11.56	0.26 ± 0.04
Strain 5	1.23 ± 0.47	12.98	0.09 ± 0.04
Strain 6	2.72 ± 0.77	13.52	0.20 ± 0.06

Table 2. Survival rate of fungal spores after treatment with EMS at a concentration of 5% and 8% (v/v) for 30, 60, 90 min

Strength of EMS (%)	Duration of treatment (min)	Name assigned to treated fungal spores	No. of colonies counted in PDA plate	Survival rate (%)
0% (Control)	Control (0 min)	Wild type	259	100
5%	30 min	$EMS_{5\%-30\ min}$	136	52.50
	60 min	$EMS_{5\%-60\ min}$	98	37.83
	90 min	$EMS_{5\%-90\ min}$	87	33.59
8%	30 min	$EMS_{8\%-30\ min}$	44	16.98
	60 min	$EMS_{8\%-60\ min}$	15	5.79
	90 min	$EMS_{8\%-90\ min}$	4	1.54

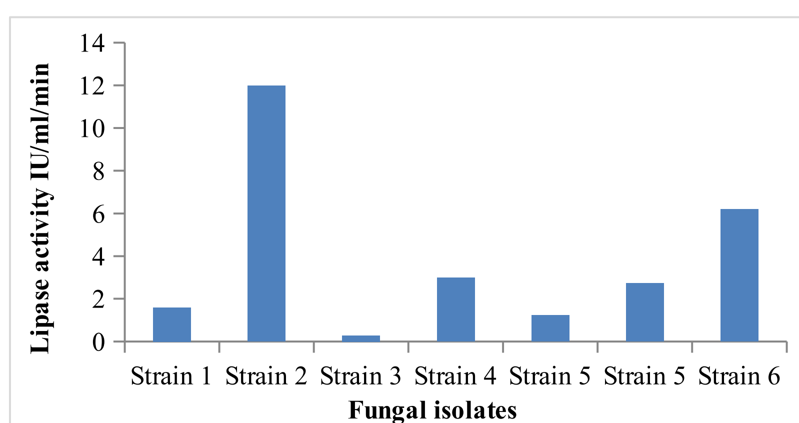

Fig. 1. Lipase activity from different fugal isolates of petrol pump soil sample at fifth day of incubation period

suppressing these regulatory mechanisms. Microbial cultures which have simple feed back inhibition, multivalent feed back inhibition and concerted feed back inhibition are used for strain improvement through random mutagenesis. Another method of improvement in the productivity is optimization of culture medium ingredients and culture conditions (3).

Lipases are the largely accepted biocatalysts that have significant applications in encouraging a variety of biochemical processes in the industry (4). They are found to be successful in catalyzing many processes related to the food, medical diagnostics, pharmaceutical, leather, dairy, cosmetic, detergent, beverage, fatty acid and paper industries (5, 6). Microbial lipases have gained industrial attention due to their activity and stability under various pH, temperature, organic solvents and metal ions (7).

Lipases can be utilized for polyurethane and fatty waste degradation (8). Lipases (EC 3.1.1.3) are universal enzymes mainly found in plants, animals, yeast, fungi and bacteria. But microbial lipases have gained more industrial attention than other sources of lipases (9, 10). Environmental genomics is a strategy used to discover unique, potentially significant enzymes as well as lipases (11, 12).

Type of mutagen and its concentration made mutagenic procedures so that mutant types may attain and further screened for enhanced activity (13). Hypersecreting strain made by strain improvement is utilized in industries to make procedure inexpensive. Strain improvement through RDT (Recombinant DNA Technology) *i.e.* site directed mutagenesis and random mutagenesis lead to higher production of desired metabolite. It has reduced the cost of industrial process in the past few years (14, 15).

It has been reported that genetically modified strain may possess various advantageous features which can be obtained by mutagenesis and reduces the cost of metabolite production (16).

There are reports on the development of optically active compounds from resolution of racemic mixtures by the activity of lipase (17). Mutation can occur naturally (spontaneous) or it can be induced by mutagens. It can be injurious or beneficial and it may be induced either through physical mutagens or chemical mutagens (1).

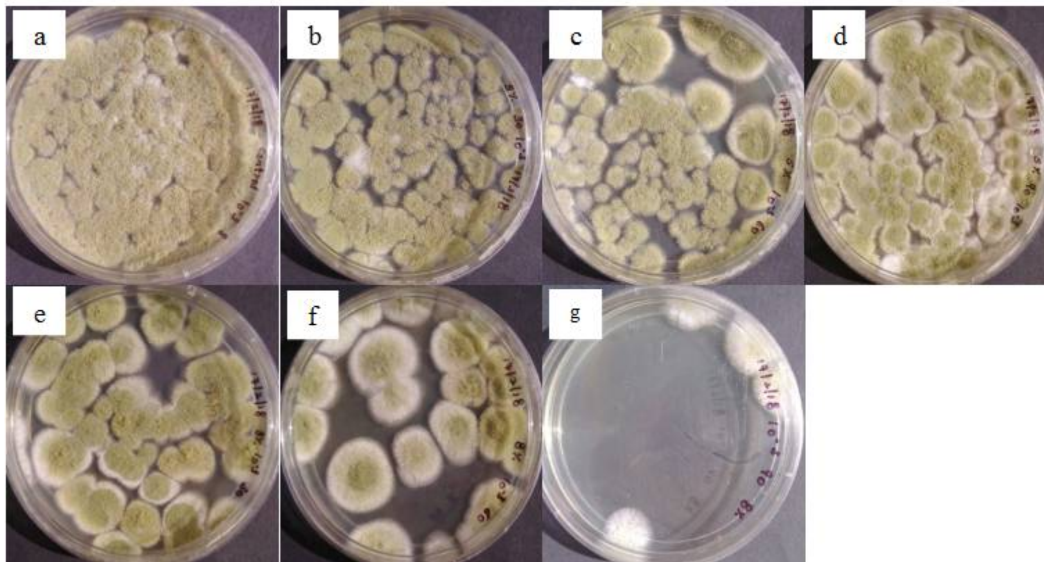

Fig. 2. PDA plates inoculated with fungal spores treated with EMS for different time intervals (**a**): Wild (untreated, 0%, 0 min); (**b**): 5%, 30 min; (**c**): 5%, 60 min; (**d**): 5%, 90 min; (**e**): 8%, 30 min; (**f**): 8%, 60 min; (**g**): 8%, 90 min

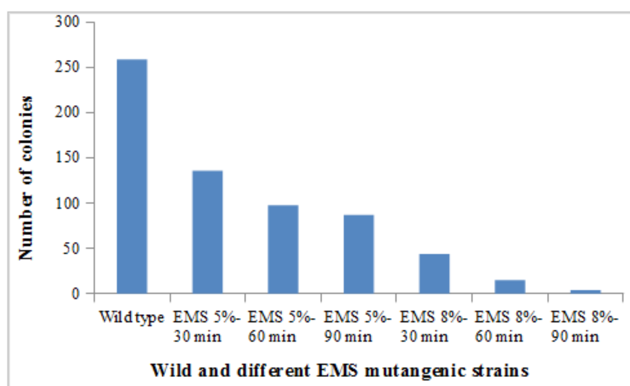

Fig. 3. Survival curve after EMS mutagenesis

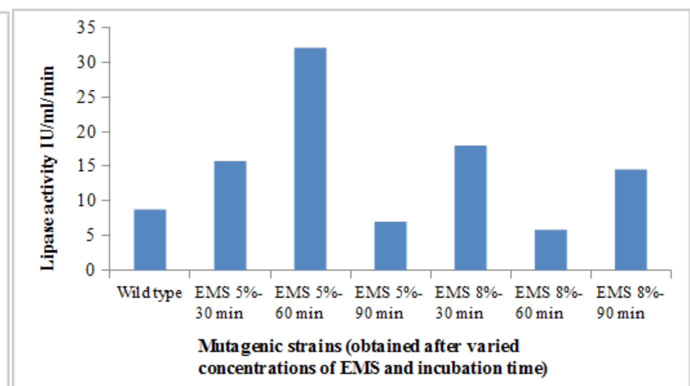

Fig. 4. Lipase activities in SmF from wild strain and EMS mutagenic strains

Table 3. Lipase production, specific activity and dry weight of EMS mutagenic fungal strains and wild strain in SmF

Incubation time of spores with EMS at varied concentration	Lipase activity (IU/ml/min)	% increase in activity	Specific activity (U/mg)	Dry weight of fungal cell pellets (g)
Wild, 0 %, 0 min	8.77 ± 3.52	100	1.02 ± 0.42	1.58
5%, 30 min	15.76 ± 3.42	179.70	1.51 ± 0.35	1.55
5%, 60 min	32.09 ± 1.84	365.90	1.99 ± 0.21	1.46
5%, 90 min	7.01 ± 0.57	79.93	0.51 ± 0.05	1.78
8%, 30 min	18 ± 2.36	205.24	1.44 ± 0.19	1.77
8%, 60 min	5.85 ± 0.48	66.70	0.51 ± 0.05	1.72
8%, 90 min	14.55 ± 0.98	165.90	1.39 ± 0.09	1.67

The demand of lipases is continuously increasing as the growth of enzyme industries. The increased demand of lipases can be achieved by improving the lipolytic potential of microbes (17). Increased lipolytic potential and capability of more lipase production can be achieved by genetic improvement of the newly isolated microbial strains through random mutagenesis. Understanding the role of lipases in industries an attempt was carried out to increase lipase production by EMS mutagenesis of natural soil fungal isolate (18).

Materials and Methods

Collection of soil sample

The soil sample was collected with sterile spatula in a sterile plastic bag from the petrol pump, Newai (Rajasthan). Sample was stored at 4ºC in refrigerator until its use.

Screening of lipase producing fungi

Six isolated strains were used for lipase production in SmF. Hyperproducer lipolytic strain 2 was further used for strain improvement

Table 4. Lipase production, specific activity and dry weight of EMS mutagenic fungal strains and wild strain in SSF

Incubation time of spores with EMS at varied concentration	Lipase activity (IU/ml/min)	% increase in activity	Specific activity (U/mg)	Dry weight of mycelium (g)
Wild, 0%, 0 min	1.77 ± 0.71	100	0.53 ± 0.19	3.462
5%, 30 min	3.01 ± 1.52	170.05	0.49 ± 0.25	3.271
5%, 60 min	2.45 ± 0.41	138.41	0.30 ± 0.05	4.142
5%, 90 min	4.34 ± 0.87	245.19	0.48 ± 0.12	4.361
8%, 30 min	3.45 ± 1.80	194.91	0.53 ± 0.26	3.963
8%, 60 min	3.06 ± 0.59	172.88	0.50 ± 0.06	3.214
8%, 90 min	7.99 ± 0.12	451.41	1.04 ± 0.02	4.229

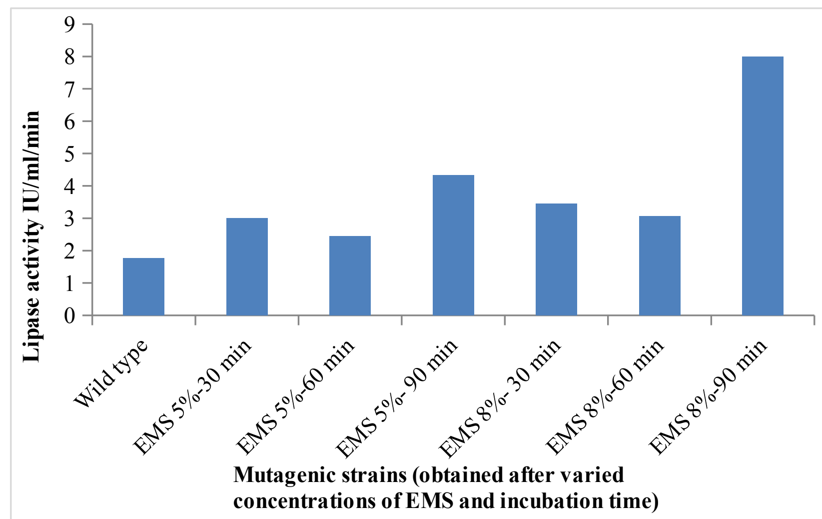

Fig. 5. Lipase activity in SSF from wild strain and EMS mutagenic strains

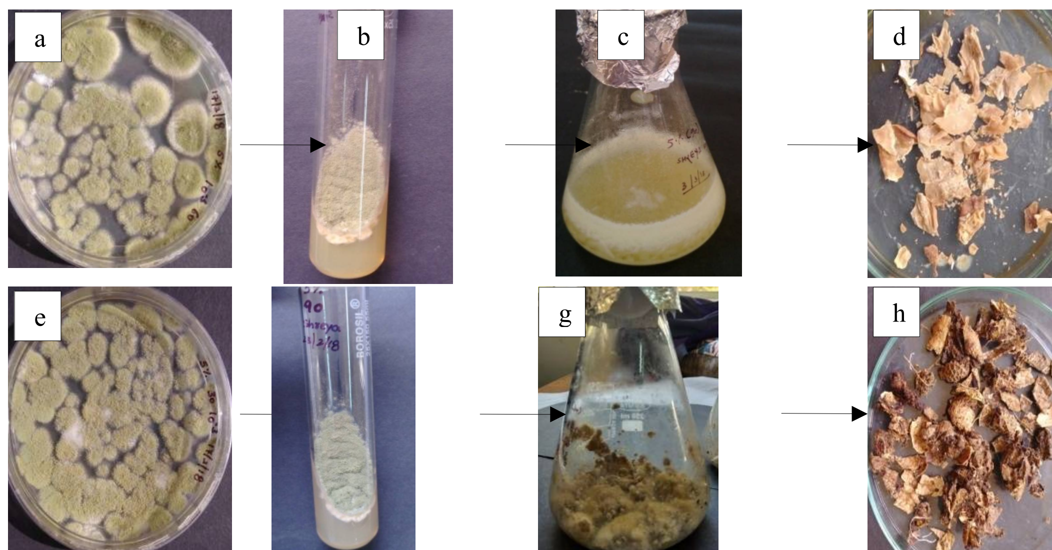

Fig. 6. Brief process of hyperproducer mutant strain EMS$_{5\%-60\ min}$ and EMS$_{8\%-90\ min}$ obtained by SmF & SSF, respectively. **(a):** Petri plate culture of isolate of EMS$_{5\%-60\ min}$ in PDA medium; **(b):** Slant culture of EMS$_{5\%-60\ min}$ in PDA medium; **(c):** growth of fungal cell pellets in production medium; **(d):** dry weight of fungus (EMS$_{5\%-60\ min}$) cell pellets obtained from SmF; **(e):** Petri plate culture of isolate EMS$_{8\%-90\ min}$ in PDA medium; **(f):** Slant culture of EMS$_{8\%-90\ min}$ in PDA medium; **(g):** growth of fungal mycelium in SSF; **(h):** dry weight of fungus mycelium obtained from SSF

through random mutagenesis using ethyl methane sulfonate (EMS). Lipase activity had been earlier described (19) and protein contents were also determined (20).

Mutagenesis using ethyl methane sulfonate (EMS)

A stock solution of 5% and 8% (v/v) ethyl methane sulfonate was prepared in sterile distilled water. Spore suspension was prepared by adding 8 ml of sterile distilled water into 10 days old slant culture. Spore suspension and EMS solution were mixed in equal volume *i.e.* 0.9 ml of spore suspension was mixed with 0.9 ml of 5% or 8% (v/v) EMS solution in three different Eppendorf tubes followed by incubation for 30, 60 and 90 min respectively, in a shaking incubator at 120 rpm at 37°C. Control tube was prepared by mixing 0.9 ml

of spore suspension with 0.9 ml of sterile distilled water followed by incubation for 30, 60 and 90 min, respectively. After specific time intervals Eppendorf tubes were removed from incubator and centrifuged at 10000 rpm for 10 min at 4ºC. Supernatant containing EMS solution was discarded and pellet was dissolved in 1 ml of sterile distilled water. After treatment of spores with mutagen, different dilutions (10^{-1}, 10^{-2}, 10^{-3}) of treated spores and wild spores (untreated) were prepared from each Eppendorf tube and each dilution was inoculated onto the PDA plates followed by incubation at 28ºC for 5 days. Number of fungal colonies in each plate was counted on 2nd day in order to prepare the survival curve (21). Survival rate was calculated by using given formula and survival curve was prepared (22).

$$\text{Survival rate} = \frac{\text{Number of test colonies}}{\text{Number of control colonies}} \times 100$$

Screening of potent mutagenic strain under submerged fermentation (SmF) and solid state fermentation (SSF)

Production media for SmF was prepared by dissolving 4 g of peptone, 0.1 g of KH_2PO_4, 0.1 g of $MgSO_4.7H_2O$, 0.1 g of $(NH_4)_2SO_4$, 0.5 g of Sucrose and 1 ml of olive oil in 100 ml of distilled water and pH was adjusted to 7. Production media was autoclaved, cooled and inoculated with spore suspension of wild and mutant fungal cultures separately. After incubation, fermentation broth was filtered and centrifuged at 10000 rpm for 10 min at 4ºC. Then, supernatant was used to determine lipase activity and protein content (23). Similarly, media for SSF was prepared by adding total 5 g of mixture of mustard oil cake, Taramira (*Eruca sativa*) oil cake, ground nut peel and soyabean bran. To this, 6.5 ml of czapek dox broth was added for moistening followed by autoclaving at 121ºC for 25 min at 15 psi. It was inoculated with spore suspension of wild and mutant fungal cultures separately. After incubation, 5% glycerol water added to all culture flasks and left for 2 h. Flasks were filtered through double layer of muslin cloth and centrifuged at 10000 rpm for 10 min at 4ºC. Supernatant was used to determine protein content and lipase activity.

Results and Discussion

The isolated six fungal strains were used for lipase production in SmF and their lipolytic and specific activities are represented in Table 1. Among all fungal isolates, strain 2 exhibited the maximum lipase activity (11.98 ± 0.46 IU/ml/min) (Fig. 1). Therefore, it was utilized for further strain improvement through random mutagenesis using EMS at a concentration of 5% and 8% (v/v). There are reports on the screening of lipolytic fungi in SmF (23).

From SmF the Strain 2 was further subjected to random mutagenesis using EMS at a concentration of 5% and 8% (v/v) for improvement in lipase productivity. Colony formation on the petri plates (Fig. 2) and the survival curve was formulated (Fig. 3). As the exposure time increased (0, 30, 60, 90 min) the survival rate was decreased. Highest survival rate (100%) was observed on PDA plate inoculated with wild strain and survival rate was lowest (1.54%) on PDA plate inoculated with fungal spores after treatment with of 8% concentration of EMS for 90 min (Table 2) (13).

It was reported that 46 to 100% lethality was obtained when spores of *Ashbya gossypii* was incubated for 1-2 h with EMS at a concentration of 5-20% (v/v) (24). Plates showing 60% mortality after treatment with 5% EMS were considered for screening of potent mutant strains. There are reports on the incubation of spores of *Aspergillus niger* with EMS at a concentration of 2 mg to 10 mg for 30-60 min (25).

Screening of potent mutant strain in SmF

Table 3 shows that best EMS mutagenic strain ($EMS_{5\%\text{-}60\ min}$) demonstrated high level of lipase activity (32.09 ± 1.84 IU/ml/min) and specific activity (1.99 ± 0.21 U/mg) as compared to lipase activity (8.77 ± 3.52 IU/ml/min) and specific activity (1.02 ± 0.42 U/mg) of wild strain (untreated spores). The best mutant strain was obtained after 60 min treatment of fungal spores with EMS at a concentration of 5% (v/v) (Fig. 4). When the mutagenic strains ($EMS_{5\%\text{-}30\ min}$, $EMS_{8\%\text{-}30\ min}$ and $EMS_{8\%\text{-}90\ min}$ strains) were incubated with EMS then activity of lipase was increased as compared to wild strain. Mutation showed significant impact on production of lipase. The dry weight of fungal cell pellet in existence with $EMS_{5\%\text{-}60\ min}$ (1.46 g) was lower than other treated fungal spores reflects that increased growth does not mean increased lipase production. Selected strain ($EMS_{5\%\text{-}60\ min}$) was used for further studies. $EMS_{5\%\text{-}60\ min}$ exhibited 365.90% increase in activity than 100% activity of wild strain.

Reports are on the comparative study production of lipase from wild and mutant strains of *Aspergillus niger* under SmF and demonstrated increased activity of mutant strain as compared to wild strain (2). Reports are on the N-methyl-N-nitro-N-nitroguanidine was found highly efficient than UV rays for strain improvement of *Aspergillus niger* for increased production (17). Reports are on the increased lipase activity by random mutagenesis of *A. niger*, *A. fumigats* and *Penicillium* sp. using EMS, UV and sodium azide (26).

There are reports stating that EMS is an ethylating agent and it can generate both GC to AT and AT to GC transition mutations (27). Use of EMS for strain improvement of *A. niger* (MBL-1511) (21). It was also demonstrated phosphate buffer pH 7.0 was used for preparation of spore

suspensions. Without treatment of spores with EMS served as control. Spores were incubated with mutagen from 30 to 150 min with 30 min of interval at room temperature and showed 133% to 232% increased lipase production as compared to the wild strains. Report are on 135% increased in lipase activity than wild strain when treated with EMS. *Pseudomonas* strain obtained after EMS mutagenesis showed two times higher lipase activity than wild strain (28).

Screening of potent lipolytic fungal strain in SSF

The best EMS mutagenic strain ($EMS_{8\%\text{-}90\ min}$) demonstrated high level of lipase activity (7.99 ± 0.12 IU/ml/min) as compared to lipase activity (1.77 ± 0.71 IU/ml/min) of wild strain (Table 4). The lipase activity was steadily increased with the rise of incubation time of spores with EMS and reached to maximum after 90 min of treatment time (Fig. 5). As the exposure time and concentration of EMS was further increased the lipase activity was also increased. The present results suggest that EMS can be used as potent chemical mutagen for increasing lipolytic activity from wild strain of fungus (Fig. 6). $EMS_{8\%\text{-}90\ min}$ exhibited 451.41% increase in activity as compared to 100% activity of wild strain. The dry weight of $EMS_{8\%\text{-}90\ min}$ strain mycelium was found to be 4.229 g as compared to dry weight (3.462 g) of wild strain.

The SmF and SSF techniques were used to determine the lipase production from mutant strain (*Aspergillus fumigatus* MTCC 9657) (29). Observation are on the incubated spores of *Aspergillus oryzae* with EMS for up to 90 min with an objective to increase enzyme production under SSF on rice bran (30).

Similar to present result, reports on the mutant strains that obtained after physical or chemical mutagenesis of fungi possess higher lipase activity than wild strain (21). Mutant strain exhibit increased activity than wild strain because of alternation of gene sequence of microorganisms (31, 32). EMS alters the base pairing due to its strong mutagenic character. Production of extracellular lipase from *Aspergillus niger* by both the SmF and SSF process (33). It was reported that SSF is an alternative to cultivate industrial lipases at economical rate by using low priced agro industrial residues as a production medium (34).

Conclusions

In present investigation, production of extracellular lipase was enhanced by strain improvement of local soil fungal isolate through random mutagenesis using two different concentrations (5%, 8% v/v) of EMS. Spores were mutagenized with EMS (5%, 8% v/v) for 30, 60 and 90 min. Lipase production was increased probably due to introduction of random mutations by nucleotide substitution by EMS. Based on the survival rate, six mutagenic strains were screened for extracellular lipase production under SmF and SSF. Among all, mutant strain named as $EMS_{5\%\text{-}60\ min}$ exhibited maximum lipase activity in SmF. Lipase activity was 365.90% more than the 100% activity of wild strain. Another mutant strain named as $EMS_{8\%\text{-}90\ min}$ exhibited maximum lipase activity in SSF. Lipase activity of mutant strain was 451.41% more than the 100% activity of wild strain. In present study, EMS was found to be effective mutagenic agent for increased lipase activity of fungi.

Authors' contribution

AKS provided the protocols for experiments and analyzed the collected data and justified the results through discussion. All the experimental work was conducted by S and assisted by R and NB.

Acknowledgements

We would like sincerely to thank Vice-Chancellor, Banasthali Vidyapith for providing research facilities. We acknowledge the Bioinformatics Center, Banasthali Vidyapith supported by DBT for providing computation support, and DST for providing networking and equipment support through the FIST and CURIE programs at the Department of Bioscience and Biotechnology. CESME, Banasthali Vidyapith supported by MHRD, Government of India under the PMMMNMTT is acknowledged for organizing the symposium.

Competing Interests

The authors declare that they have no competing interests.

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