➔DOI : 10.15740/HAS/AJBS/11.1/91-94
e ISSN-0976-8343 | ■ Visit us : *www.researchjournal.co.in*
AFBSAH-2016 ASIAN JOURNAL OF BIO SCIENCE
Volume **11** | Issue 1 | April, 2016 | 91-94

RESEARCH PAPER

Combined mutagenic improvement of *Bacillus licheniformis* SK7 for cost-effective protease production

SANJAY KUMAR YADAV[1], **POONAM SINGH**[1], KASHYAP KUMAR DUBEY[2] AND BHANU P. SINGH[2]

[1]Department of Molecular and Cellular Engineering, Jacob School of Biotechnology and Bio-Engineering, Sam Higginbottom Institute of Agriculture, Technology and Sciences, ALLAHABAD (U.P.) INDIA
[2]Microbial Biotechnology Laboratory, University Institute of Engineering and Technology, M.D. University, ROHTAK (HARYANA) INDIA
Email : drpoonam.singh1@gmail.com

Article Info : Received : 10.12.2015; Accepted : 09.02.2016

The strain improvement of developed *Bacillus licheniformis* SK7 was achieved with the combination of physical and chemical agents *i.e.*, UV + NTG + EMS by gradually mutation and positive selection. In case of UV light 99 per cent killing for successful selection of mutants was 6 min and 10 cm distance from source, while in case of EMS and NTG the concentration found effective was 200 µM, 30 µM, respective for 15 min. The mutant *Bacillus licheniformis* SK7 SN 43 was successfully developed and found stable having higher production of protease (662 U/ml) under optimized medium and physical conditions of fermentation than the wild type *Bacillus licheniformis* SK7 (472 U/ml) under the same conditions of fermentation.

Key words : Mutagenesis, NTG, UV light, Strain improvement, Protease

How to cite this paper : Yadav, Sanjay Kumar, Singh, Poonam, Dubey, Kashyap Kumar and Singh, Bhanu P. (2016). Combined mutagenic improvement of *Bacillus licheniformis* SK7 for cost-effective protease production. *Asian J. Bio. Sci.*, **11** (1) : 91-94 [Special Issue of AFBSAH-2016].

INTRODUCTION

The bacterial strain improvement has been used for the production of industrially important enzymes. The strain improvement process involves classical mutagenesis methods and r-DNA technique. The classical mutagenesis techniques were found to be superior for the strain improvement. These methods include classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, γ-rays, UV rays, etc., and chemical mutagens such as NTG, EMS, EtBr, etc. (Parekh *et al.,* 2000). This method of strain improvement was cost effective; the genus *Bacillus* was most important micro-organism for commercial protease production (Haki and Rakshit,2003; Bapiraju *et al.,* 2004 and Koncerova *et al.,* 1984). The use of mutation and selection to improve the productivity of strains was valuable tool for strain improvement of many enzyme-producing organisms (Kaur *et al*., 1998). Mutations lead to variations in random genes. These genes can have a positive or negative influence in the physiological and morphological characteristics of an organism (Jeremy and Simon, 2010). Changes in the random genes can drastically affect the type of protein, end product produced, yield of produce protein (Snyder and Champness, 2003). Mutation was the primary source of all genetic variation and has been used extensively in industrial improvement of enzyme production (Ghisalba *et al*., 1984; Sidney and Nathan, 1975). Improved strains can be achieved by inducing genetic variation in the natural strain and subsequent screening. In recent years new procedures such as

rational screening and genetic engineering have begun to make a significant contribution to this study but mutagenesis and selection- so-called random screening was still cost effective procedure and reliable method for strain development and getting the stable improved mutant strains (Rowlands, 2002). The present study was mainly focused on the strain improvement of *Bacillus licheniformis* SK7, through physical and chemical mutation methods alone in a combination of physical and chemicals agents. The stable high producing promising strains *Bacillus licheniformis* SK7 will be used in protease production in an economic method and will be used in various industrial applications.

RESEARCH METHODOLOGY

Bacillus licheniformis SK7 was previously isolated by the authors. All the chemicals used were analytical grade.

Medium and fermentation conditions :

The fermentation medium consisted of ferric chloride 1.16 g/lit., skimmed milk 10.0 g/lit., glycerol 1.0 g/lit. and fish meal 7.6 g/lit. The fermentation of *Bacillus licheniformis* SK7 and further mutants was carried out in the above said medium at pH at 9.0, 220 rpm 48 hrs of incubation at 37°C.

Mutagenesis :

Ethylmethane sulphonate (EMS) was added to the spore suspension at different concentrations and incubated for 15 min in shake flasks at 37°C. and N-Methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) was added to the spore suspension at different concentrations and incubated for 15 min in shake flasks at 37°C. To terminate mutagenesis, the spore suspensions were done by the method Stonesifer and Baltz (1985), Miller (1983). The respective spore suspensions (20 µl) were spread into Petri dishes containing media (g/l, w/v): casein, 10.0; yeast extract, 1.0; KH_2PO_4, K_2HPO_4, $CaCl_2$, 0.1; $MgSO_4.5H_2O$ 0.05 and agar, 15.0, pH 9, and incubated at 37ºC. The plate was exposed to UV light (UV light source: TFP 20 M UV screen, 6.times.15 W, 213 nm from Vilber Lourmat, France) the radiation dose (exposure time and intensity) which led to the desired killing rate of *Bacillus licheniformis* SK7 cells was determined in preliminary tests. The kill rate was between 50 per cent and 99 per cent. Cells mutagenized by following the method Cruger and Cruger (2004) and Bockrath *et al.* (1987).

Assay of proteolytic activity :

Determination of the most potent isolates was performed by estimating the proteolytic activity in the previously obtained supernatants by using the method proposed by Meyers and Ahearn (1977) with some modifications. The protein content of the supernatant was estimated according to (Lowry *et al*., 1951). One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzed casein to produce 1µg tyrosine per ml per minute under the above assay conditions. Standard calibration curves were done for protease using tyrosine (Sigma).

RESEARCH FINDINGS AND ANALYSIS

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Isolation and properties of *Bacillus licheniformis* SK7 mutants by UV light :

Optimization of UV mutation distance vs per cent killing :

The agar plates after spreading of *Bacillus licheniformis* SK7 for 10 minutes time from 5 cm to 50 cm. The results showed that the killing was maximum when the plates were kept for 10 min at a distance of 10 cm from UV source *i.e.*, 98 per cent and it was observed that the killing percentage decreased with the increase in distance between the agar plate and the source of UV

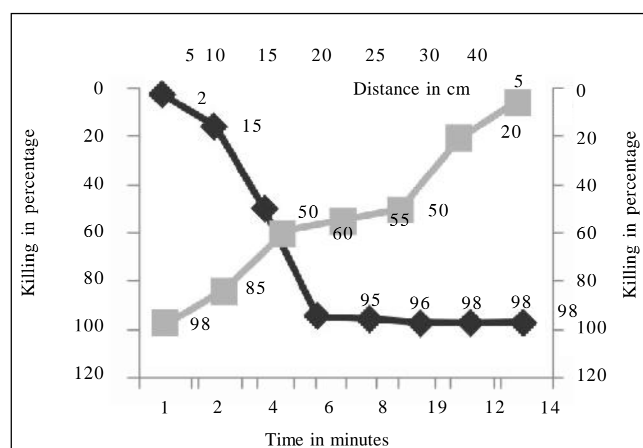

Fig. 1: Effect of distance and time of UV source from plate on *Bacillus licheniformis* SK7

light (Fig. 1). From the above results the distance of UV light was set 10 cm as the killing is 60 per cent so as to give more time of exposure of UV light to *Bacillus licheniformis* SK 7 spread agar plates, the time of UV exposure was optimized and found that after the 6 min the killing reached to 98 per cent, which is optimal for the selection of mutants (Fig. 1) (Gupta *et al*., 2002).

Optimization of ethyl methanesulfonate concentration:

The spore suspension of *Bacillus licheniformis* SK7 was subjected to treat with EMS at various concentrations ranging from 10µM to 300 µM. It is noticed that after the EMS concentration of 200µM the killing percentage was 99 per cent suitable for the selection of mutants (Fig. 2) (Ralph, 2003).

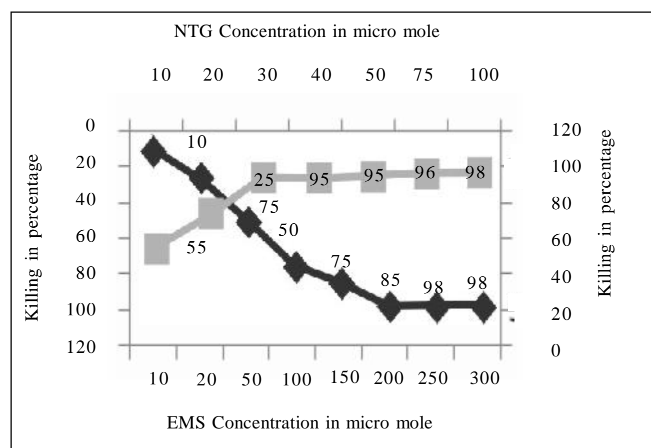

Fig. 2: Effect of various concentration of EMS and NTG on killing of *Bacillus licheniformis* SK7

Optimization of -methyl-N'- nitro-N-nitrosoguanidine (NTG) concentration :

The spore suspension of *Bacillus licheniformis* SK7 was subjected to treat with NTG at various concentrations ranging from 10 µM to 100 µM. It is noticed that after the NTG concentration of 30 µM the killing percentage was 99 per cent suitable for the selection of mutants (Fig. 2) (Hopwood *et al.,* 1985).

Selection of mutants of *Bacillus licheniformis* SK7 :

From the treatment of the above optimized treatments the mutants was selected on the basis of morphological alternation (Fig. 3) and screening on casein agar plate. Various mutants were screened for each treatment. The mutant UV 35 was found the most productive (502 U/ml) and the colony was having mucous like secretion among mutants screened from UV mutagenesis. The mutants EMS 3, NTG 27 and NTG 73 were found more productive and were used for further combined mutagenesis experiments having protease production of 526 U/ml 535 U/ml and 556 U/ml, respectively. The further mutation of NTG 73 with EMS and UV resulted in the increase in protease production and the selected mutants SM 22, SNE 17 and SN 43, the production of protease was found 572 U/ml, 605 U/ml and 662 U/ml in fermentation media, respectively (Table 1, Fig. 3) (Nadeem *et al*., 2010).

Table 1: Comparison of various selected mutants of *Bacillus licheniformis* SK 7 for protease production in control media

Sr. No.	Islolate name	Protease production in U/ml
1.	*Bacillus licheniformis* SK 7	472
2.	EMS 3	526
3.	NTG 27	535
4.	UV 35	502
5.	NTG 73	556
6.	EMS + NTG (SM 22)	572
7.	NTG + UV (SN 43)	662
8.	NTG + UV + EMS (SNE 17)	605

Fig. 3: Comparison of various selected mutants of *Bacillus licheniformis* SK7 on morphological basis and casein agar plate method for selection

Conclusion :

The developed mutant *Bacillus licheniformis* SK 7 SN 43 was tested more than six times for their stability and productivity. The stability of this mutant was verified after three months sub- culturing. The isolated mutant showed higher protease production than the wild strain and high production was always reproduced in the experiments repeated afterwards. So the combinations of various mutagenic methods were most successful in obtaining the stable mutant with higher significantly higher production of protease.

LITERATURE CITED

Bapiraju, K.V.V.S.N., Sujatha, P., Ellaiah, P. and Ramana,T. (2004). Mutation induced enhanced biosynthesis of lipase. *Afr. J. Biotechnol.,* **3**(11): 618-624.

Bockrath, R., Ruiz-Rubio, M. and Bridges, B.A. (1987). Specificity of mutation by UV light and delayed photoreversal in umuC-defective Escherichia coli K-12: a Targeting intermediate at pyrimidine dimers. *J. Bacteriol.,* **169** (4): 1410-1416.

Cruger, W. and Cruger, A. (2004). *Textbook of industrial microbiology*, 2nd Ed. (Biotechnology) Sinauer Associates, Sunderland, U.S.

Ghisalba, O., Auden, J.A.L., Schupp, T. and Nuësch, J. (1984). The rifamycins: properties, biosynthesis and fermentation. In E.J. Vandamme (Ed.), *Biotechnology of industrial antibiotics*. pp. 281-327. Marcel Dekker, Inc. NEW YORK, U.S.A.

Gupta, R., Beg, Q. and Lorenz, P. (2002). Bacterial alkaline protease molecular approaches and industrial applications. *Appl. Micribiol. Biotechnol*., **59**:15-32.

Haki, G. and Rakshit, S.K. (2003). Development in industrially important thermostable enzymes a review. *Biores. Technol.,* **89** : 17-34.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. and Schrempf, H. (1985). *Genetic manipulation of Streptomyces: a laboratory manual.* The John Innes Foundation, NORWICH, UNITED KINGDOM.

Jeremy, W.D. and Simon F.P. (2010). *Molecular genetics of bacteria,* 5th Ed. A John Wiley & Sons, Ltd., Publication.U.K.

Kaur, M., Dhillon. S., Chandhary, K. and Singh, S. (1998). Production, purification and characterization of thermostable alkaline protease from *Bacillus polymyxa*. *Indian J. Microbiol*., **38**: 63-67.

Koncerova, H., Vachova, L. and Chaloupka, J. (1984). Mutants of *Bacillus* megatereum with altered synthesis of an exocellular neutral proteinase. *Folia. Microbiol*., **29**: 99-103.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., **193**: 265-275.

Meyers, S.P. and Ahearn, D.G. (1977). Extracellular proteolysis by *Candida lipolytica*. *Mycologia*., **69**: 646-651.

Miller, J. (1983). Mutational specificity in bacteria. *Annu. Rev. Genet.*, **17**: 215–238.

Nadeem, M., Qazi, J.I. and Baig, S. (2010). Enhanced production of alkaline protease by a mutant of *Bacillus licheniformis* N-2 for dehairing. *Braz.Arch. Biol. Technol.,* **53**(5): 1015-1025.

Parekh, S., Vinci, V.A. and Strobel, R.J. (2000). Improvement of microbial strains and fermentation processes. *App. Microbiol. Biotechnol*., **54**: 287-301.

Ralph, K. (2003). *Fundamentals of biochemistry, cell biology and biophysics*. Vol. **2**, Prokaryote genetics.

Rowlands, R.T. (2002). Industrial strain improvement: mutagenesis and random screening procedures. *Enzyme & Microbial Technnol*., **6** (1): 3-10.

Sidney, P. C. and Nathan, O.K. (1975). Methods in enzymol. *J.Microbiol.,* **3**: 26.

Snyder, L. and Champness, W. (2003). *Molecular genetics of bacteria*, 2nd Ed., ASM Press, Washington, D.C., U.S.A.

Stonesifer, J. and Baltz, R.H. (1985). Mutagenic DNA repair in *Streptomyces*. *Proc. Nat. Acad. Sci. USA,* **82**: 1180–1182.

AFBSAH-2016 ★★★★★ International Conference on ★★★★★ JSBB, SHIATS
ADVANCING FRONTIERS IN BIOTECHNOLOGY FOR SUSTAINABLE AGRICULTURE AND HEALTH

11th **Year**
★★★★★ **of Excellence** ★★★★★