CHAPTER 2 STRAIN IMPROVEMENT

Abstract

Strain improvement by mutagenesis and selection is a highly developed technique and it plays a central role in the commercial development of microbial fermentation processes. This chapter includes studies on strain improvement of *Aspergillus niger* NCIM 563 for enhanced phytase production under submerged fermentation conditions. The use of classical approach for strain improvement of *A. niger* NCIM 563 included a combination of physical (UV) and chemical mutagenesis (EtBr and Hydroxyl amine) which resulted in obtaining two mutants P16 (*A. niger* NCIM 1359) and P33 (*A. niger* NCIM 1360) which were found to be superior to parent strain as they produce 156 and 95 U/ml phytase activities on 10.5\textsuperscript{th} day and 7\textsuperscript{th} day, respectively as compared to 68 U/ml of phytase activity by parent on 11th day. Mutants *A. niger* NCIM 1359 and *A. niger* NCIM 1360 showed increase in activity up to 230 % and 145 %, with increase in productivity up to 2.4 and 2.2 times that of parent respectively.

1. Introduction

Importance of microbial genetics was realized in 1940s during the global bloom of penicilin production. At the beginning the studies pertained to creation and assessment of mutants, however, effortlessness of the technique and its ability to change phenotypic expression of characters lead to unhindered exploitation of microbial genetics. Increase in productivity at industrial scale by thousand folds has been an outcome of
mutagenesis moreover it is also responsible for flourishing of the fermentation industry. Strain improvement plays a central role in the commercial development of microbial fermentation processes. The current practice of strain improvement by mutagenesis and selection is a highly developed technique (Parekh et al, 2000). Mutagenic procedures can be carried out in terms of type of mutagen and its dosage or concentration so as to obtain mutant types that may be screened for improved activity. Microorganisms do not overproduce any metabolite naturally, unless made to do so. For the strain to be used at industrial scale, it must be hypersecreting; so as to make the process economical. Strain improvement carried out through mutagenesis followed by screening or using recombinant DNA technology has lead to higher industrial productivities and simultaneous reduction in production costs in the last few years (Vinci and Byng, 1999). Strain improvement can be achieved by creating strains (i) with ability to utilize inexpensive complex raw materials; (ii) increasing product concentrations or reducing by-product interferences; (iii) extracellular secretion of product; (iv) insensitivity to product inhibition; (v) reduction in fermentation time etc (Parekh et al, 2000).

Two ways by which microbes can create new characters are mutation and genetic recombination. Mutations can either be spontaneous or induced. Mutations can be harmful or beneficial for the organism the former being eliminated by selection. Mutagenesis can be carried out by either physical or chemical methods, while protoplast fusion, conjugation, transformation and recombinant DNA technology are other means of genetic recombination. Mutagenesis approach is widely applied at industrial level to alter concentrations of the metabolite of interest (Parekh et al, 2000), along with other applications such as studies on metabolite pathways,
search for new/novel compounds etc. some of the most potent mutagens include hydroxyl amine, ethyl methyl sulphonate, methyl methane sulphonate (MMS), nitrosoguanidine (NTG), 4-nitroquinolone-1-oxide and ultraviolet light (UV) (Table 1).

Table 1: Mutagens and their mode of action (Parekh et al 2000).

<table>
<thead>
<tr>
<th>Mutagen</th>
<th>Characteristic of mutation induced</th>
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<tr>
<td>X rays, Gamma rays</td>
<td>These ionizing radiations causes strand breakage in DNA leading to deletions and structural changes in DNA.</td>
</tr>
<tr>
<td>Ultraviolet rays</td>
<td>These short wavelength radiations cause pyrimidine dimerization and crosslinking leading to transversions, deletions and frame shift mutations in DNA.</td>
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<tr>
<td>5-chloro/uracil, 5-bromouracil</td>
<td>Results in faulty pairing leading to AT to GC transitions.</td>
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<tr>
<td>2-aminopurine deaminating agents</td>
<td>Causes errors in DNA replication.</td>
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<tr>
<td>Hydroxyl amine</td>
<td>Causes deamination of cytosine leading to GC to AT transition.</td>
</tr>
<tr>
<td>Nitrous acid</td>
<td>Causes deamination of A, G and C leading to bidirectional translation and deletion.</td>
</tr>
<tr>
<td>N-methy, N’-nitro</td>
<td>Causes methylation leading to GC to AT transition.</td>
</tr>
<tr>
<td>N-nitroso guanidine</td>
<td>Causes alkylation of C and A leading to GC to AT transition.</td>
</tr>
<tr>
<td>Methyl methyl sulphonate, Ethyl methyl sulphonate</td>
<td>Causes alkylation of C and A leading to GC to AT transition.</td>
</tr>
<tr>
<td>Ethidium bromide, acrydine dyes</td>
<td>Causes intercalation between two base pairs leading to frameshifts and microdeletions.</td>
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Generally, mutants are obtained by subjecting the organisms to the mutagenic agent for a defined period of time known as kill time (95-99.9 % kill) followed by culture in media, screening and selection for the metabolite of interest (Simpson and Caten, 1979). This mutant screening process ensures isolation of best quality of mutants sans its low success rate (Baltz, 1999).

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) is a major storage form of phosphorus in many cereals and legumes, representing 18-88 % of total phosphorus content (Dvorakova et al, 1998; Reddy et al, 1982). Phytic acid acts as an antinutrient as it binds to essential dietary minerals such as calcium, zinc, magnesium and iron decreasing their bioavailability (Ford et al, 1978). Phytases (EC 3.1.3.8 for 3-phytase and EC 3.1.3.26 for 6-phytase) hydrolyse the phytic acid in a stepwise manner to get inositol phosphates, myo-inositol and inorganic phosphate. A large number of micro-organisms produce phytases (Selle & Ravindran, 2007). Since phytase is absent or not present in sufficient amounts in the gut of monogastric animals (Bitar & Reinhold, 1972; Common et al, 1989) the supplementation of phytase in poultry feed is essential to improve phosphorus bioavailability. It also reduces the phosphorus pollution in the areas of livestock production due to decomposition of underutilized phytate (Ravindran et al, 2001). Thus because of its industrial importance the ultimate objective here has been to produce this enzyme at a cost effective level.

Many experiments and field trials have shown that 500 to 1000 units of phytase can replace approx. 1 g inorganic phosphorus supplementation and reduce total phosphorus excretion by 30–50 % (Kemme et al, 1997, Lei et al, 1993, Liu et al, 1997, Yi et al, 1996). Phytic acid is used in pulp industry as an anti-oxidant to prevent aging of paper. Use of phytase in
phytic acid degradation during pulp and paper processing, seems to be an eco-friendly way of waste management (Liu et al., 1998). Importance of soil microbes for increasing availability of phytate-phosphorus to plant roots has been suggested by several researchers (Richardson et al., 2001; Tarafdar & Marscner, 1995; Singh and Satyanarayana, 2010).

Several efforts were made to obtain phytase overproducing strains of industrial value, however, success for acidic phytase (phyB) from A. niger are yet to be achieved. A high level of functional expression of A. fumigatus phytase gene was achieved in Pichia pastoris (Rodriguez et al., 2000). With the aim of improving cost-effective phytase production, the gene phyA from A. niger var. awamorii ALKO243 was cloned which resulted in several fold overproduction of phytase (Piddington et al., 1993). The phyA gene encoding heat-stable phytase was cloned from A. fumigatus and overexpressed in A. niger (Pasamontes et al., 1997). Chelius and Wodzinski (1994) carried out strain improvement of A. niger for phytase production using UV as a mutagen and resistance to hygromycin B as the selection criteria. However, they could not obtain significant changes in acidic phytase (phyB) production levels as compared to 3.3 fold increase obtained for phytase I (phyA).

Aspergillus niger phytase (PhyA) has been used as a feed supplement to improve the bioavailability of phytate phosphorus in monogastric animals. Due to inability of phytase enzyme to survive at pelleting temperatures, several workers across the globe have been working on improving the thermal stability and other biochemical properties of phytase. Yan Liao and coworkers (2013) used site directed mutagenesis approach to improve catalytic efficiency and thermostability of phytase of A. niger N25 wherein they achieved a 20 % improvement in
thermostability in mutants when compared to the wild type strain. They were also able to improve the catalytic efficiency of phytase significantly.

Natuphos, Phytase Novo™ (Cao et al, 2007), Finase (Meittinen-Oinonen et al, 1997) etc. are some commercial phytases obtained from different organisms. All available phytase preparations used as feed additives today are of fungal origin and produced by submerged fermentation (SmF) and are active at pH 5.0. Fungal phytases have serious shortcomings, especially with regard to their sensitivity to heat and inactivation under low pH conditions generally present in the stomach (Afinah et al, 2010). These disadvantages need to be alleviated in the use of fungal phytase as feed enzyme in animal diet. This work is directed towards realizing these aims while simultaneously increasing phytase production rates.

2. Materials and Methods

2.1 Organism

The organism used Aspergillus niger NCIM 563 was used throughout the experiments. It was obtained from NCIM Resource Center, India and was maintained on Potato Dextrose Agar (PDA) slants at 4 °C.

2.2 Medium and culture conditions

Fermentation medium for phytase production contained (per 100 ml): rice bran 1 g; glucose 5 g; NaNO₃ 0.86 g; KCl 0.05 g; MgSO₄·7H₂O 0.05 g; FeSO₄·7H₂O 0.01 g, pH 5.5 before sterilization. Fermentation medium (100 in 250 ml Erlenmeyer Flask) was inoculated with 1 % (v/v) of spore suspension (1 x 10⁷ spores/ml) prepared by suspending the spores from 7 day old sporulated slant of A. niger NCIM 563 grown on PDA in 10 ml of
sterile saline containing 0.01 % (v/v) Tween 80 and incubated at 30 °C at 200 rpm. Samples were removed after every 24 h and checked for pH, growth, total residual reducing sugar, extra cellular protein and phytase activity.

2.3 Selection of 3 log kill mutant dose by kill curve

The spore suspension (10^7/ml) was obtained from 7 days old slant of A. niger NCIM 563 grown on PDA, by scraping in 10 ml sterile saline containing 0.01 % Tween 80. The spore suspension was treated with mutagen for different time intervals. The appropriately diluted samples were then plated on PDA plates. The Colony Forming Unit per ml (CFU/ml) was used to calculate the 99% kill time.

2.4 Analytical methods

2.4.1 Phytase activity

Phytase activity was measured using 100 mM Glycine-HCl buffer, pH 2.5 at 50 °C for 30 min as described earlier (Bhavsar et al, 2008). One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 μmol phosphorus/min under standard assay conditions.

2.4.2 Protein estimation

Protein concentration in the culture filtrate was determined using the method of Lowry et al (1951) using Bovine serum albumin as standard.

2.4.3 Sugar content

Total residual reducing sugar concentration was estimated by DNSA method (Miller 1959).
2.5 Isolation of A. niger mutant

The spore suspension (10^7 spores ml) was treated with mutagens, both physical (UV) and chemical viz. Ethidium bromide (0.1 mg/ml) and Hydroxyl amine (0.1 %) for different time intervals. The appropriately diluted samples were then plated on PDA plates. The Colony Forming Unit per ml (CFU/ml) was used to calculate the 99 % kill time. Mutants obtained were selected by spreading treated spore suspension on slightly modified phytase screening medium (PSM) agar plates containing 0.5 % calcium phytate and 0.05 % NaNO₃. The selection of mutants was based on the enhanced zone of calcium phytate hydrolysis.

3. Results

3.1 Kill Curve

Kill curve calculation is the basic step of mutagenesis experiments. It helps researchers’ determine the concentration or time period of exposure of the mutagenic agent in order for the mutagenesis to succeed. Time interval for 99 % kill was obtained to be 45 min (Fig 1).

3.2 Isolation of phytase producing A. niger mutant

Mutants were selected on the basis of small compact colony with larger zone of hydrolysis on calcium phytate plate as compared to parent strain (Fig 2). All the positive mutants were evaluated for phytase production using rice bran–glucose–salt medium in shake flask condition.
Mutants P-16 and P-33 were found to be superior to parent strain as they produce 156 and 95 U/ml phytase activities on 10.5\textsuperscript{th} day and 7\textsuperscript{th} day, respectively as compared to 68 U/ml of phytase activity by parent on 11\textsuperscript{th} day (Fig 3). P16 and P33 mutants isolated showed increase in activity up to 230 \% and 145 \%, with increase in productivity up to 2.4 and 2.2 times that of parent respectively (Table 2).

Figure 1: Kill curve of Fungal spores

Figure 2: Plate based screening
The morphological patterns of the mutant strains were evaluated by using microscopy. Mutant and parent strains showed striking differences in mycelial morphology, sporangium shape and sporangium size (Fig 4). Another remarkable difference among these strains was delay in the sporulation time of the mutant strains as compared to parent. Parent strain
showed sporulation on 4th day whereas that of mutant strains was obtained on 7th day.

Figure 4: Morphological variations (Upper row: Sporangium morphology a) Parent; b) P-16; c) P-33 Lower row: Mycelial morphology d) Parent; e) P-16; f) P-33)

4. Discussion

Though several organisms have been reported to produce phytase, the expression levels are far from the idea of commercialization with an economical point of view. Combinatorial mutagenesis studies were conducted, using different combinations of ethidium bromide or hydroxyl amine with that of ultraviolet light exposure, for obtaining hypersecretory mutant strains. The mutants were selected based on the enhanced zone of hydrolysis of phytate on phytase screening medium. However, the method is unable to differentiate between phytase activity and acid production. Hence, all the positive mutants were quantified and confirmed for phytase production using the statistically optimized media for the parent strain in shake flask condition. Among the hypersecretory
mutants, the mutant *A. niger* NCIM 1359 exhibited the highest phytase productivity (14857 U/l/d) and improved the yield 2.4 fold as compared to the productivity obtained from parent strain. Interestingly, another mutant *A. niger* NCIM 1360 exhibited equally improved productivity (13571 U/l/d) and yield (2.2 fold) with reduced fermentation time (7 days). These observations could have tremendous value addition to phytate feed-conversion and environmental aspects of phytate biology.

Strain improvement has its own pros and cons. Even if mutants with the desired phenotype are isolated, there is no guarantee that the mutation has occurred in the gene of interest also low mutation frequency in the desired gene is another difficulty associated with strain improvement. Overcoming these shortcomings and exploitation of recent developments in gene cloning and recombination can be applied to study the site and type of mutagenesis. From a simpler viewpoint, however, we can reasonably assume that genetic variability amongst parent and mutant strains exist if morphological differences are seen through mutagenesis studies. In the present study, we exploit this observation. Low yield, high production costs, and lack of desirable characteristics in the currently available commercial phytases have limited its widespread use (Krishna and Nokes 2001). Thus there is a need for identifying novel phytases with high yield and improved desired enzymatic characteristics. The results of mutagenesis show a remarkable improvement in phytase production rates which is suggestive of its potential for industrial application. *A. niger* mutants outperform the phytase production rates in comparison to other organisms, viz., *Sporotrichum thermophile* with 10,100 U/l (Singh and Satyanarayana 2006) and 12,500 U/l (Singh and Satyanarayana 2008) for two different media formulations and recombinant *Escherichia coli* with 2,250 U/l (Sunitha et al, 1999). Currently *A. niger* is “Generally Recognized as Safe (GRAS)” in food and feed applications (Bhavsar et
al, 2011). Along with the high yield, pH tolerance, and temperature stability characteristics, it appears to be a viable option when compared to other available commercial phytase supplements.
5 References


