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STRAIN IMPROVEMENT BY CLASSICAL MUTAGENESIS FOR L-ARGININE PRODUCTION

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Abstract

Improvement of the antibiotic yield of industrial strains is invariably the main target of industry-oriented research. The approaches used in the past were rational selection, extensive mutagenesis, and biochemical screening. These approaches have their limitations, which are likely to be overcome by the judicious application of recombinant DNA techniques. Efficient cloning vectors and transformation systems have now become available even for antibiotic producers that were previously difficult to manipulate genetically. The genes responsible for antibiotic biosynthesis can now be easily isolated and manipulated.

Keywords: Classical mutagenesis, rDNA technology, Strain improvement.

Introduction

A vast range of products and services are extensively provided my microorganisms. As there properties like mass cultivation, speed of growth ,use of cheap substrates (which in many cases are wastes) and the diversity of potential products proved that they are useful .Their ability to readily undergo genetic manipulation has also opened up almost limitless further possibilities for new products and services from the fermentation industries.

It is not new that these are mostly used in industrial processes. From centuries through selective breeding of microbes improving of commercial and technical capabilities of microbial strains have been practiced. In making specialty foods and fermented beverages (such as alcohol, sake, beer, wine, vinegar, bread, tofu, yogurt, and cheese), specific strains of bacteria and fungi isolated by chance have been employed to obtain desirable and palatable characteristics. Genetic recombination methods are represented by sexual or parasexual crosses in fungi and conjugation.however it is performed

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by photoplast fusion in both organis [1, 2].

The specific micro organisms are generally isolated from environment which involved the random screening of large number of isolates. On the other way, suitable micro organisms are acquired from culture collections. These microorganisms irrespective of there origin need to be subsequently modified in order to improve their properties for industrial use by strain improvement. Even though molecular genetic improvement is just strating to become a practical reality, the next important scientific and technological advance is already appearing on the horizon, challenging researcher's imagination and creativity [3].

Strain Improvement

The science and technology of genetically manipulating and improving microbial strains, in order to enhance their metabolic capacities for biotechnological applications are referred to as strain improvement. It is not simply the modification for over production of bioactive compounds. It should also be viewed as making the fermentation process more cost-effective. Some of the traits unique to fermentation process that make a strain "improved" are ability to

(a) Assimilate inexpensive and complex raw materials efficiently.
(b) Alter product ratios and eliminate impurities or byproducts problematic in downstream processing.
(c) Reduce demand on utilities during fermentation (air, cooling water or power draws).
(d) Excrete the product to facilitate the product recovery.
(e) Provide cellular morphology in a form suitable for product separation.
(f) Create tolerance to high product concentrations.
(g) Shorten fermentation times
(h) Overproduce natural products or bioactive molecules, not synthesized naturally, for example, insulin.

Need for Strain improvement

To produce novel compounds of commercial interest microbes like fungi, bacteria, actinomycetes are isolated from natural surroundings that live in soil or water are not ideal for industrial use. In general wild strains cannot make the product of commercial interest at high enough yields to be economically viable. In nature, metabolism is carefully controlled to avoid wasteful expenditure of energy and the accumulation of intermediates and enzymes needed for their biosynthesis. This tight metabolic and genetic regulation, and synthesis of biologically active compounds, is ultimately controlled by the sequence of genes in the DNA that program the biological activity.

The sequence of these genes must be altered and manipulated to improve microbial strains[4] . In essence, microbial strain improvement requires alteration and reprogramming of the DNA in a desired fashion to shift or bypass the regulatory controls and checkpoints. Such DNA alterations enable the microbe to devote its metabolic machinery to produce the key metabolic enzymes and increase product yields. In some cases simple alteration in

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DNA can also lead to the structural changes in a specific enzyme that increases its ability to bind to the substrate, enhance its catabolic activity, or make itself less sensitive to the inhibitory effects of a metabolite. On the other hand, when the changes are made in the regulatory region of the gene (such as promoter site), it can lead to deregulation of gene expression and overproduction of the metabolite.

Depending primarily on the kind of improvement desired from the microbe which is the outcome of the strain selection. For instance, increased product yield that involves the activity of one or more genes, such as enzyme production, may be enhanced simply by increasing the gene dosage. Molecules such as secondary metabolites [5] and antibiotics that are complex in structure and require a coordinated as well as highly regulated biosynthetic process, however, may require a variety of alterations in the genome to derive a high yielding strain.

Methods of strain improvement

Genetically altering the strain so that it overproduces the desired product is an important step in industrial microbiology. Three such general procedures have been used successfully; mutation, genetic recombination and gene cloning. Often two or three of these techniques can be used in tandem to obtain a stable improved strain.

Historically mutant screening was the first systematic method to improve industrial strains. Starting in 1944, the strain NRRL-1951 of Pencillium species was used as the [6] parent strain for a program of mutation and selection by several groups of investigators. The results were remarkable and the lineage of strain improvement for the pencillin production is given below Table 1.

Strain improvement in penicillin production.

Year	Penicillin strain	Origin	Yield (g/l)
1929	Penicillium (Fleming)	Chance contamination	0.01
1941	NRRL-832	Isolated in Belgium	0.04
1943	NRRL-1951	Isolated from a melon	0.15
1944	X-1612	X-ray mutant of NRRL 1951	0.30
1945	Q-176	UV mutant of X-1612	0.55
1949	49–133	Spontaneous mutants of Q-176[a]	1.2
1990	Commercial strains	Nitrogen mustard mutants of 49–133[a]	>7.0

[a]Several steps of mutation and selection were used to obtain this overproducing strain.

Table 1: Showing several steps of mutation obtain by overproducing strain.

The significance of this strain improvement program is that today it is possible to produce enough penicillin [7] at a low cost to treat anyone who needs the antibiotic, whereas before only a few serious cases could be treated at a high cost.

Genetic recombination of advantageous mutations from several mutant strains is a useful procedure for strain improvement. It allows one to combine advantageous mutations from different sources. Also, genetic recombination makes possible the removal of deleterious secondary mutations. For example, when a culture

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is mutagenized and then a bacterium is selected which overproduces the desired product; the bacterium may also contain mutations that interfere with growth. By backcrossing the mutant strain into wild type, it is possible to screen for strains that contain the useful mutation without the deleterious one. This procedure of "cleaning up" the strains is particularly important when multiple mutation steps are employed.

Gene manipulation is the third and most recent technique of strain improvement. This method requires a good understanding of the molecular genetics and biochemical pathway that is involved in the biosynthesis of the desired product. Gene manipulation can be used to overcome rate-limiting reactions by increasing the production of specific enzymes. This can be obtained by cloning the gene and increasing its copy number, by altering promoter strength and ribosome binding sites, and eliminating undesirable properties such as product inhibition. In addition, gene manipulation can be used to generate new products by combining genes from different microorganisms.

Strain Improvement by Classical mutagenesis

The simple concept of laboratory evolution – random mutagenesis and screening or selection of the "fittest" – is the basis principle of classical mutagenesis [8]. The insertion of random point mutations into DNA has been used to generate mutant strains since industrial biocatalysis began. UV and X-ray irradiation or mutagenic chemicals such as nitrous acid, formic acid or hydrazine have been used to generate production strains in industry as a way of so called "classical strain improvement"; random mutagenesis of the whole genome and subsequent screening for better performing variants. Despite successes in the rational and targeted engineering of fermentation strains, the primary means of improving even well characterized and mature industrial organisms remain classical strain improvement. This is because most whole cell phenotypes are by nature complex, and it is difficult to model or identify which genes in the dynamic cellular machine may be limiting.

The first key step of the classical approach is the generation of a mutant population. Alterations at the gene level in a microorganism are typically achievable by subjecting the organism to a mutagen. There are a variety of mutagens which include X-ray, Ultraviolet light, Hydroxylamine, and N-methyl-N'-nitro-N-nitrosoguanidine [9]. Different mutagens have different mechanisms of act including base transitions, base deletions, or base additions. It is essential that at the beginning of a strain improvement program that different mutagens are tested to identify the one that is most effective in generating the desired diversity in a mutation population. Sometimes, it might be necessary to switch to a different mutagen as strain improvement progresses. The key to the success in generating a diversified mutant population is to identify the ideal condition for mutation of the microbe. "Under mutagenesis" [10] leaves a large number of mutation survivors without genetic alteration and therefore results in screening a large number of strains identical to the parent. "Overmutagenesis" [11], on the other hand is more likely to produce a population in which survivors accumulate multiple mutations,

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significantly reducing the chance of finding desired mutation. Therefore, the aim of an ideal mutagenesis strategy is to identify the dose of the mutagen that maximizes the frequency of survivors that contain only one yield affecting mutation.

Mutation survivors are segregated, followed by growth on either solid agar media or liquid media for the development of individual colonies. Single colonies are chosen randomly and fermented in small scale fermentation vessels. When seeking mutants with increased productivity, it is more likely to find mutants with a small increment in productivity instead of a blockbuster high-producing mutant. However in order to identify mutants with a small titer increment, the screening system must be sufficiently consistent and sensitive to reduce the chance of finding false mutants.

Once fermentation is complete, the metabolite is extracted from the fermentation culture and quantified. Mutants with increased metabolite productivity are considered potential hits and are re-evaluated in multiple replications to determine statistically if they are better than the parent strain. Mutants that demonstrate improvement during the confirmation process are used as parents for a new round of mutation and screening.

Materials and Methods

Strain Bacillus Subtilis OUT 8103 was brought from Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh, India. Arginine-producing mutants were derived from Bacillus Subtilis OUT 8103.

Mutagenesis of Bacterial strain Bacillus Subtilis OUT 8103

Sterile Nutrient broth, active Bacillus Subtilis OUT 8103 culture, boiling test tubes, NTG, Citrate buffer, minimal A buffer, Sterile Saline water, Sterile Nutrient Agar plates.

A loop full of the active culture is inoculated into 5ml nutrient broth under sterile conditions and incubated overnight in a shaking incubator at 37 ˚C and 200 rpm. 50 µl of the above grown culture is added to each of the six sterile boiling tubes that contain 5 ml nutrient broth and incubated for 8 – 10 hours (until the O.D reaches 0.8) in a shaking incubator at 37 ˚C and 200 rpm. Centrifuge all the above six microbial suspensions at 8000 rpm for 15 minutes and discard the supernatant. Take six tubes and name them as control, 200µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml & 600 µg/ml and add NTG (Stock concentration 1mg/ml) to all the tubes except control at labeled concentrations [12]. Make the volume to 5 ml in each tube with citrate buffer. Incubate the tubes at 37 ˚C in an incubator for 45 min. Collect the cell pellet of six tubes by centrifuging at 8000 rpm for 15 min. Wash the cell pellet twice by using minimal A buffer (K_2HPo_4, KH_2Po_4, $(NH4)_2Po_4$ and Sodium Citrate) solution. Finally suspend the cell pellet in 2 ml minimal A buffer solution. Take 0.1 ml of each of the above microbial suspension and serially dilute it up to 10^{-4} dilutions. Take six sterile minimal media agar plates and label them as control, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml & 600 µg/ml. 0.1 ml of the above microbial suspension from each of the 10^{-4} dilution tube is spread plated onto the respective Minimal Media plate. Incubate the plates at 37 ºC

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for 24 hours. Count the number of colonies on each plate. Compare the number of colonies to that of the control plate. The concentration at which the number of colonies would be reduced to 80%, when compared to number of colonies on the control plate will be considered as optimized concentration.

Isolation and Purification of Arginine producing mutants.

Sterile nutrient broth, active Bacillus Subtilis OUT 8103, NTG, Sterile Minimal A buffer, Sterile Saline Water, Sterile Nutrient Agar Plates, Boiling Tubes, Sodium Citrate Buffer.

A loop full of the active culture is inoculated into 5ml nutrient broth under sterile conditions and incubated overnight in a shaking incubator at 37 ˚C and 200 rpm. 50 µl of the above grown culture is added to a boiling tube that contains 5 ml nutrient broth and incubated for 8 – 10 hours (until the O.D reaches 0.8) in a shaking incubator at 37 ˚C and 200 rpm. Centrifuge the above microbial suspensions at 8000 rpm for 15 minutes and discard the supernatant. Add NTG at optimized concentration, determined in the previous experiment to the boiling tube and make the volume of the boiling tube to 5 ml. Incubate the tube at 37 ˚C in an incubator for 45 min. Collect the cell pellet of the tube by centrifuging at 8000 rpm for 15 min. Wash the cell pellet twice by using minimal A buffer (K_2HPo_4, KH_2Po_4, $(NH4)_2Po_4$ and Sodium Citrate) solution. Finally suspend the cell pellet in 2 ml minimal A buffer solution. Take 0.1 ml of each of the above microbial suspension and serially dilute it up to 10^{-4} dilutions. Take the Minimal media plate in which the analogue is present at the minimum inhibitory concentration of Bacillus Subtilis OUT 8103, optimized in the first experiment. 0.1 ml of the above microbial suspension from 10^{-4} dilution tube is spread plated onto the above Minimal Media plate. Incubate the plates at 37 ºC for 24 hours. The resistance colonies obtained thereby will be picked and purified on the same medium for 4 – 5 generations. The purified colonies will be checked for Arginine production.

Results and Discussion:

Microorganisms employ a variety of mechanisms to tightly control the synthesis of primary metabolites such as amino acids. Therefore, the metabolites are produced only in small quantities sufficient for essential cellular activities. One of the mechanisms used to prevent unnecessary overproduction of essential metabolites is feedback regulation. There are two types of feedback regulation: one is feedback inhibition in which the end product of a biosynthetic pathway inhibits the action of an enzyme in the way; the second is feedback repression, which is inhibition of enzyme synthesis by the end product. Industrial microorganisms used for the production of amino acids [13]are often developed to be less sensitive to feedback regulation by using analogs for selection. Such anti metabolites of amino acids often inhibit cell growth by interfering with the cells' machinery used for making those amino acids. Mutants deregulated in feedback control are resistant to the toxic effect of the analog and typically, overproduce the metabolite to which the analog is antagonistic. Therefore, analog-resistant mutants overproduce the end product if the toxicity is the result of the fact that

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the analog mimics the control properties of the end product and the site of resistance of the resistant mutant is the site of control by the end product.

Arginine could be produced by mutants with a defective regulatory mechanism of it's biosynthesis and such regulatory mutants could be found among a group of analogue resistant mutants [14]. Hence, the first step of the classical strain improvement for Arginine production is the identification of an Arginine analog that can mimic the control properties of the end product. Arginine hydroxamate, D-Arginine and Homo Arginine are some of the Arginine structural analogs, out of which Arginine hydroxamate is found to be the most effective. Therefore, in order to achieve high Arginine production titers we had isolated mutants of wild Bacillus Subtilis OUT 8103 cells, resistant to Arginine Hydroxamate and these mutants had shown Arginine production levels of 1.2 – 1.5mg/ml.

Estimation of Arginine production.

Seed media: Glucose – 2%, Peptone – 1%, Yeast Extract – 1%, Nacl – 0.5%, Initial P^H -6.52
Adjusted P^H – 7.2 (Before sterilization). Autoclave done at 121^0C for 15 min. Glucose was sterilized separately, after cooling the media 2% glucose was added to seed media.

Production media: $(NH_4)2SO4$ - 3%, KH2PO4 -0.05%, K2HPO4 – 0.05%, MgSO4 – 0.025%, Caco3 - 2%, Initial pH-6.84, Adjusted P^H – 7.2 (Before sterilization). Autoclave done at 121^0C for 15 min. Glucose was sterilized separately, after cooling the media 10% glucose was added to production media.

Inoculate the desired colony into seed media and then into production media. For every 24 hours estimation of L-Arginine through Sakaguchi test is to be done. Prepare Arginine standard solution by dissolving 0.1grams of Arginine in 10 ml of sterile water. Prepare working standard by diluting 100µl of standard Arginine in 9.9ml of sterile water. Label boiling tubes at different concentration range 200 µl, 400 µl, 600 µl, 800 µl, 1000 µl, 1200 µl, 1400 µl, 1600 µl, & 2000ul. Add double distilled water to make up to 4ml. Add 1ml of KOH to each tube and mix gently. Add 2ml of 0.1% naphthalene and then add 1ml of 40% urea mix gently. All tubes keep at 4^0 C for 10 min. Then add 1ml Potassium hypobromite and gently mix. Take O.D. values by UV Spectrophotometer at 540nm. Standard Graph will be constructed and concentration of Arginine in culture is checked at different time points in Figure 1.

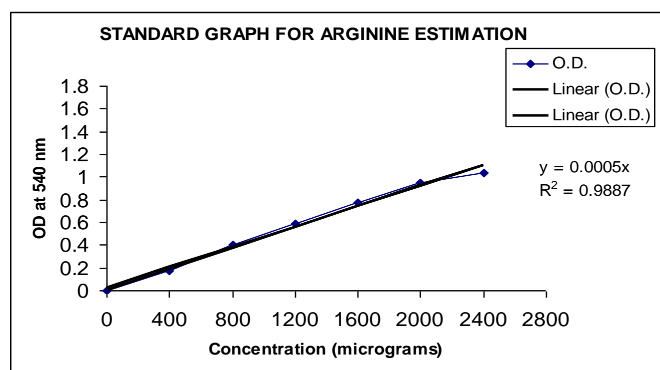

Table 2: Standard graph for arginine estimation

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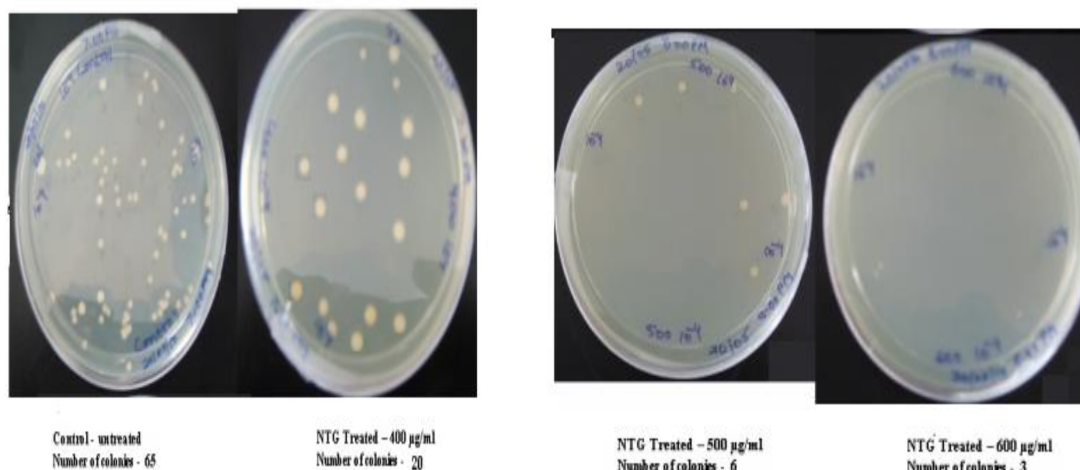

Figure:2

Figure:3

Figure 2 & Figure 3 Showing survival of organism at different mutagen concentration.

From the above pictures, it is evident that the survival rate of wild cells decreases with the increase of mutagen concentration. The plate in which microbial cells treated with 500 µg/ml mutagen (NTG) were plated contains only 15% survived cells in figure 1 and figure 2. These few survived cells could be the effective mutants. In the next plate, very few cells had survived and therefore with such concentrations of mutagens, the chances of obtaining mutants will diminish. Hence, the optimum mutagen concentration for the treatment of Bacillus Subtilis OUT 8103 microbial is determined as 500 µg/ml of NTG.

Isolation and Purification of Arginine producing mutants:

Active microbial cells are treated with the mutagen and are grown in the presence of arginine hydroxamate, at

previously determined growth inhibitory concentrations. After 3 – 5 days of incubation, the colonies grown are considered as mutant colonies and purified on the same medium.

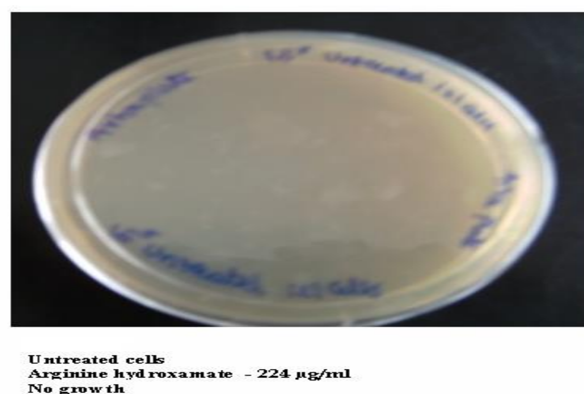

Figure 3: Plate showing no growth of microbes in arginine analogue medium.

In the above plate where wild Bacillus Subtilis OUT 8103 microbial cells were plated, there was no growth as the Arginine analogue figure 3 – Arginine

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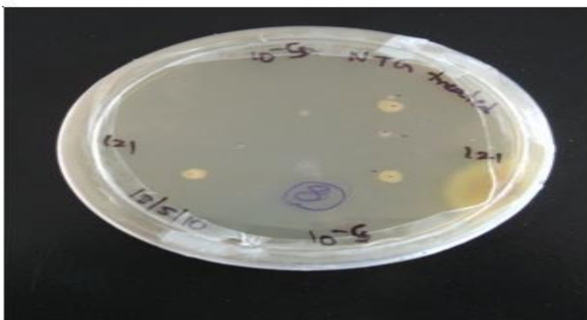
Hydroxamate is toxic to the wild cells at 224 µg/ml concentration.

Name of the colony	OD AT 540 for 72 hours	Concentration
Arghx4	0.34	1.52 mg/ml
Arghx6	0.38	1.58 mg/ml
Arghx7	0.39	1.62 mg/ml
Arghx13	0.28	1.20 mg/ml

Table 3: Concentration of arginine produced by different colonies.



NTG treated cells
Arginine hydroxamate - 224 µg/ml
8 colonies obtained

Figure 4: Number of colonies observed at arginine hydroxmate concentration at 224ug/ml.

In the above plate figure 4 where mutated Bacillus Subtilis OUT 8103 microbial cell were plated, eight colonies of mutated Bacillus Subtilis OUT 8103 were able to grow and these colonies are known as resistant Bacillus Subtilis OUT 8103 cells of Arginine Hydroxamate. They have the ability to overproduce the Arginine.Arginine biosynthesis in microbial cells is regulated by feedback repression mechanism. When wild strain cells are grown in the presence of Arginine hydroxamate, a structural analogue of Arginine, the cells will stop the synthesis of Arginine due to the feedback repression of the pathway by Arginine hydroxamate and therefore Arginine is unavailable to cells and thereby growth is inhibited.Random mutagenesis of wild Bacillus Subtilis OUT 8103 cells, followed by selection of resistant colonies of Arginine hydroxamate allows us to isolate only those mutated cells in which cells machinery is modified so that they can overproduce Arginine. Feedback repression in these cells will be lifted off.

Estimation of Arginine production:

Only four of the eight colonies were purified and tested for Arginine production.Shake flask cultures of the resistant mutants will be checked for Arginine production every 24 hours.Estimation was done by the spectrophotometric [15] assay of the cultures which is based on sakaguchi reaction.

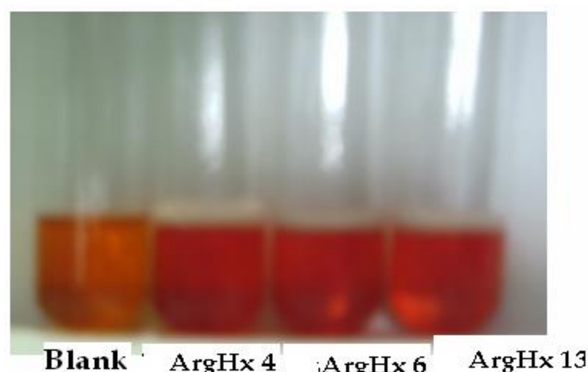


Figure 5: Spectrophotometric assay of Arginine hydroxamate resistant mutants.

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Estimated Arginine production values of Arginine hydroxamate resistant mutants

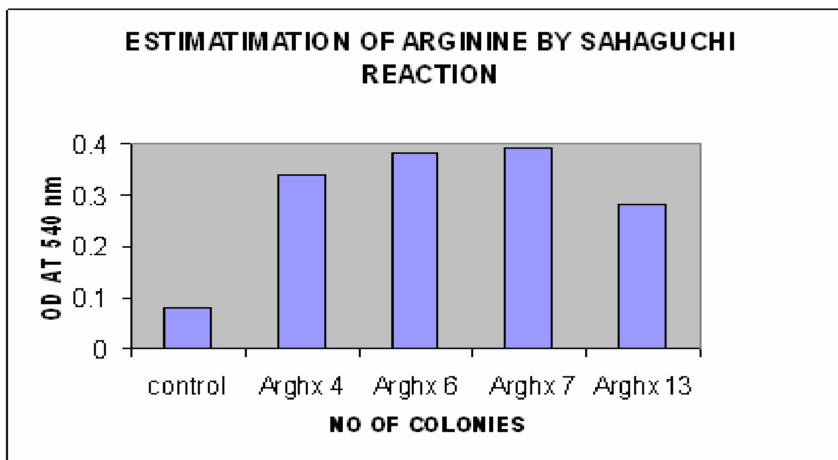

Figure 6: Graphical representation of Arginine production titer.

In comparison to the wild strain which produces neglizable amount of Arginine, the four colonies – Arghx4, Arghx6, Arghx7 and Arghx13 in figure 6 had shown high Arginine production titers i.e. in the range of 1.2mg/ml – 1.65 mg/ml. Out of the four colonies; Arghx 7 produced highest levels of Arginine i.e 1.62mg/ml.

Conclusion:

Classical strain improvement has been practiced for more than 50 years and a long history of success. In view of the long history , it continues to be the primary strain improvement strategy for any newly established strain improvement program. Eventhough genetic engineering can provide an enormous potential for strain improvement in industrial microorganisms, Classical strain improvement would definitely be the good choice as it doesn't require the prior knowledge of the metabolite biochemical pathway, regulation or

transport. Particularly, Classical strain improvement is a robust technique for the improvement of primary metabolites production in microorganisms. Ideally, the classical approach and targeted approaches (rDNA technology) should be integrated to create synergistic effect for rapid strain development.

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