MICROBIAL STRAIN IMPROVEMENT FOR OVERPRODUCTION OF INDUSTRIAL PRODUCTS

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Abstract

Microbes generate a wide range of products that are of interest to human beings. These products are produced in low concentrations because microbe regulates its expenditure of energy efficiently to maximise its growth. By using metabolic and genetic engineering techniques these products can be overproduced. Strain improvement has been a hallmark of industrial processes for a long time because it is necessary to make the industrial processes more efficient and cost effective. Overproduction of primary metabolites and secondary metabolites is a complicated process therefore a prerequisite of strain improvement is having knowledge of microbe's physiology and pathway regulatory controls. However, current advances in gene manipulation techniques have made it easier to modify the strain. This review highlights different techniques and strategies that can be utilised to manipulate microorganisms into generating maximum titre of the desired industrial product.

Keywords: Primary metabolites, Secondary metabolites, Gene manipulation, Mutation.

1. Introduction

Biotechnology industry is basically based on utilising microbes, plants and animal cells to obtain a diverse range of products. Microorganisms generate a wide variety of products as part of their metabolism that are of huge benefit for humans day to day use. Some of these

products include antibiotics, enzymes, organic compounds, biofuels, etc. In nature the microbial metabolism is controlled to avoid wasteful expenditure of energy thus microbes produce these metabolites in low concentrations; however, prerequisite for any industrial scale biotechnological process is a microbial strain which generates high amounts of any desired industrial product. This tightly regulated genetic and biological system is ultimately controlled by nucleotide sequences therefore genetic modifications need to be made to the microbial genome since they don't inherently produce high amount of products. Thus, the strain is subject to numerous physical, chemical or genetic techniques to overproduce the product of interest by alteration and reprogramming of the DNA to shift or bypass the regulatory checkpoints. As shown in Figure 1, this new discipline of strain improvement integrates metabolic engineering with other disciplines such as; systems biology i.e. systems of biological components, synthetic biology and evolutionary engineering while considering upstreaming process that includes strain improvement, midstreaming process that includes fermentation, to downstreaming process that involves separation and purification, as well. Implementation of such techniques is already underway, a good example of which is the generation of Escherichia coli strains with capacity of producing L- valine and L-threonine with exuberance (Lee & Kim, 2015).

The strategy of improving microbial strains is quiet old and is referred as the so called 'old biotechnology' (Parekh, Vinci & Strobel, 2000). Few examples of old biotechnology are included in production of fermented foods and beverages employing specialised strains (Manfredinii & Cavellera, 1983). Early studies on microbial strain improvement can be dated back to the Second World War era when there was a widespread demand for antibiotics, particularly Penicillin. Hence the first work on genetic manipulation began on *Penicillium chrysogenum*. Present records show that since its original isolation, the yield of penicillin production by P. chrysogenum has been improved by approximately 500 times (Rowlands, 1984). Today the production of different enzymes, amino acids, proteins, drugs, antibiotics, etc. at a large scale is owed to strain improvement (Demain & Davis, 1998).

An improved microbial strain encapsulates enhanced ability to (1) efficiently utilise complex raw materials (2) eliminate the production of undesired by- products (3) improved extracellular delivery of by product (4) diminish toxicity of the product to ensure minimal cell death (5) minimising fermentation time (6) overproduce indigenous or foreign products (Saxena, 2015). The purpose of this review is to describe various strategies involved in

microbial strain improvement projects, what entails strain improvement procedures and screening methods for improved host strains. However, the strategies and concepts can be used for the production of therapeutic proteins from mammalian cell lines (Vinci & Byng, 1998).

2. Strategies for Microbial Strain Improvement

To develop an improved strain a proper blueprint should be developed which should employ some or all of the following strategies.

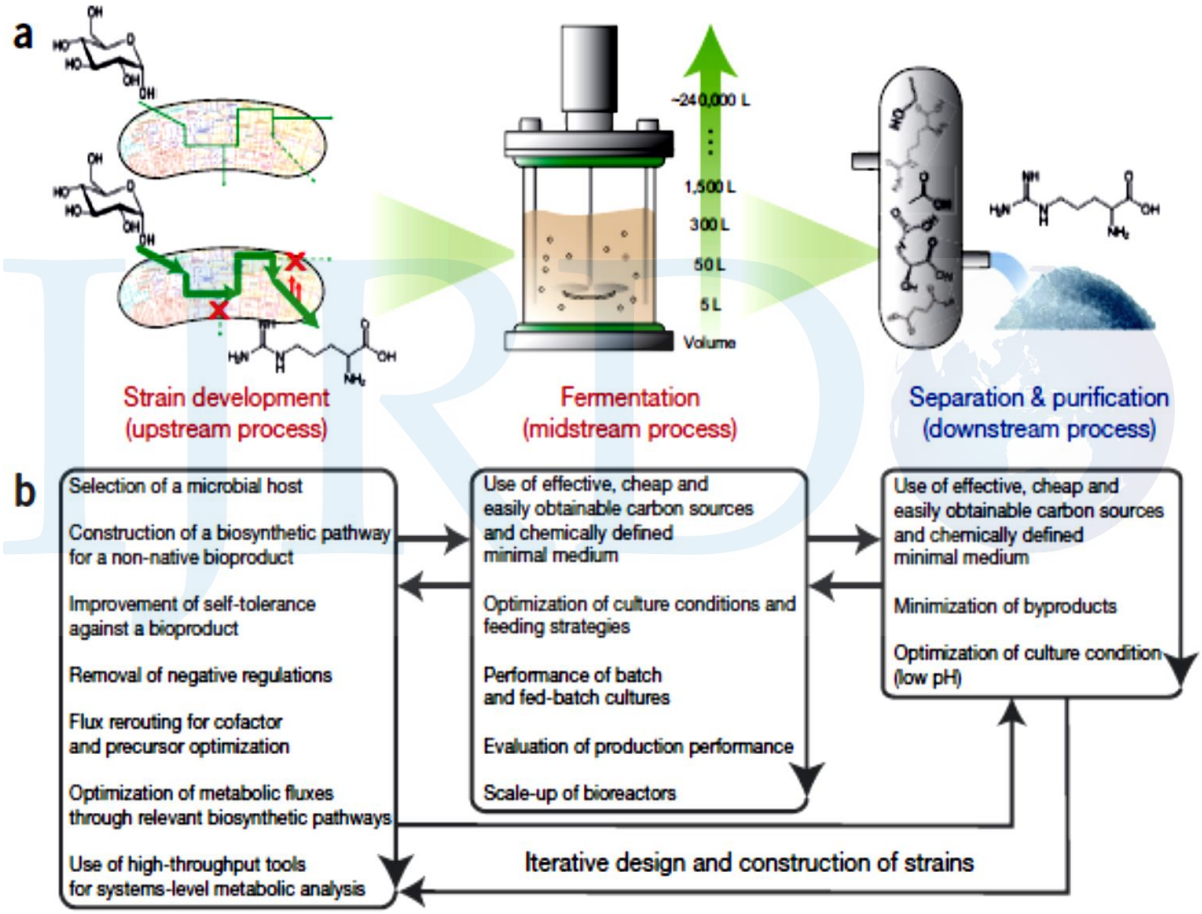

Figure 1. Interactive design of upstream, midstream and downstream processes.

2.1 Project design

When designing a project for strain improvement, various technical, economical, legal considerations need to be made. This step is usually applied in private sectors but is mostly neglected in academic sector. Once the choice between products of interest has been made, techno-economic analysis needs to be carried out with various candidate microbial strains.

The microbial analysis should include the type of fermentation; whether it's aerobic or anaerobic fermentation, its cheap carbon source and whether the fermenter used is batch or continuous or fed- batch fermenter while considering down streaming equipment as well (Zhuang & Herrgard, 2015). Various strategies can be used depending on the nature of the product, but whatever strategy is used product titre, yield and productivity should be properly estimated (Lee, Kim, Jang, Choi & Lee, 2011). For example for industrial fermentation fed batch fermenters are preferred over batch fermentation (Croughan, Konstantinov & Cooney, 2015) because of the enhanced productivity, yield and flexibility of fermentation process as well as less chances of contamination (Lee & Kim, 2015).

Apart from the technical factors the economic factors include the addition of high cost chemical inducers (IPTG – isopropyl thiogalactopyronoside) in the expression system for high yield, productivity and titre of product (Van Dien, 2013). Secondly, another aspect is the requirement of chemical modification by the precursors to yield high amounts of product. Lastly, a suitable geographical site should be selected for the manufacturing of product by fermentation (Lee & Kim, 2015).

2.2 Selection of microbial strain

Escherichia coli and *Saccharomyces cerevisiae* are the most commonly used workhorses for metabolic engineering since there is a huge availability of tools. In certain situations, other group of organisms may prove more useful, so there is a need to explore new strains for engineering. The ability of the organism to utilise carbon feedstock of choice should also be considered when selecting a strain (Lee & Kim, 2015). The emergence of novel tools to make a microbe more prone to genetic manipulation has made the process easier (Guan, Dunham, Caudy & Troyanskaya, 2010). The ability of new techniques like CRISPR and Cas9 based systems has allowed gene manipulation of those organisms that were once intractable to it (Tong, Charustani, Zang, Weber & Lee, 2015). A person has to make a choice between making an organism more susceptible to gene manipulation or choose another organism that is easily engineered. Other factor like the cost and ease of upstreaming, midstreaming and downstreaming processes should be contemplated.

2.3 Metabolic pathway reconstruction

In some cases the formation of product of interest is absent in the microbe of choice, therefore, this production pathway needs to be added to the organism by inspecting the

candidate enzymes or genes through genome and metagenome analysis (Lee et al., 2011; Shin, Kim, Kim & Lee, 2013). Obviously, this strategy is not required in organisms producing the desired product. However, this strategy is of increasing significance because of our interest in the production of products that are not natural or have inefficient production in hosts.

Heterologous pathways can be introduced into the host based on biological knowledge or genomic and cheminformatic analysis (Shin et al., 2013). Development of these pathways increases the host's capacity to produce multiple chemicals and drugs (Lee & Kim, 2015) including, antibiotics or their derivatives (Jiang & Pfeifer, 2013), certain acids (Borodina et al., 2015; Yu, Xia, Zhong & Qian, 2014), etc. Once the pathway has been constructed it can be optimised by expression optimisation or codon optimisation (Yim et al., 2011) or increasing/altering the enzyme specificity (Shin et al., 2013) etc.

2.4 Increasing tolerance to product

Microbes have a proper regulated system that prevents the wasteful expenditure of energy, thus the metabolites are produced in low amounts. Once the organism is modified to overproduce a certain product it is absolutely necessary to expand the tolerance of that strain to the product. This strategy can be adopted at any point in strain development (Ling, Teo, Chen, Leong & Chang, 2014). When it is done in early stages it will open up metabolic fluxes thus increasing the tolerance. A prerequisite level of product, before this step is adopted, is 50 to 80% of the level that begins to inhibit cell growth (Lee & Kim., 2015)

Serial culturing with increasing concentration of product at each step is used for developing a tolerant strain. This is then followed by screening of strains that survive increased concentrations (Utrilla et al., 2012). By continued repetition of this process the tolerance of the strain can be increased. If there are no other ways of increasing strain tolerance one can always design the process coupled with the continual removal of product (Lee & Kim, 2015).

2.5 Removing negative regulatory pathways

Microbial cells have optimised their energy expenditure by maximising their growth and preventing the overproduction of metabolites that effect their growth. Typical circuits that limit overproduction are transcriptional attenuation and feedback inhibition.

Transcriptional attenuation can be prevented by erasing the genes coding for repressors or replacing the enzymes genes with constitutive ones. Feedback inhibition can be prevented by inducing site directed mutagenesis to regions responsible for negative regulation. If the sites responsible are unspecified two strategies can be applied. Firstly, homology based random mutagenesis (in which a gene similar to the gene of interest is added through a vector and replaces the gene of interest by recombination) followed by screening at enzyme level. Secondly, the host can be made tolerant to increased product concentration. Thus removal of negative regulatory pathways is essential (Lee & Kim, 2015).

2.6 Rerouting fluxes to optimise precursor availability

Cofactors play a major role in metabolic reactions and their availability should be taken into account for metabolic engineering. Some examples of cofactors include NADH, NADPH, ATP and coenzyme A (CoA). Metabolic fluxes rerouting is usually a major factor for optimisation of precursor availability (Lee & Kim, 2015). Different approaches for cofactor availability can be adopted. Gene knockout is the most significant method, by which we knockout the competing genes. Gene knockdown is another approach which uses small regulatory RNAs and is useful when gene knockout of competing genes is not an option (Na et al., 2013; Yoo, Na & Lee, 2013).

2.7 Optimising metabolic fluxes towards product formation

Metabolic fluxes need to be optimised to remove any bottlenecks and to shift the flux towards product of interest formation. This needs to be performed under conditions similar to the final industrial fermentation conditions (Lee & Kim, 2015). This step should be performed in fed batch mode, because industrial fermentation involves the use of fed batch cultures (Lee, 1996). By doing so, we can prioritise further metabolic engineering towards increased product formation.

2.8 Optimising microbial culture conditions

Chemically defined media are the first choice for any laboratory scale or industrial scale fermentations. However cost of the media is often considered over any other aspect as long as the product titre or yield remains unaffected. Thus is important to produce an industrial strain that is efficient even when using impure cost effective substrates (Lee & Kim, 2015).

2.9 Gene modification to further enhance production

Once all other approaches have been carried out, other gene manipulation targets can be identified to further enhance the product formation. This step involves different approaches such as; high throughput genome scale engineering (Song, Lee & Lee, 2015), omics-based approaches (Park, Lee, Kim & Lee, 2007), *in-silico* simulations (Lewis, Nagarajan & Palsson, 2012), etc. The current issues for wide application of this strategy include the transformation inefficiencies of strains apart from *E.coli* and inadequacy of screening procedures for mutant strains overproducing desired product (Lee & Kim, 2015).

2.10 Scale-up fermentation of developed strain

Once the strain has been developed, it should be authenticated in a pilot plant scale fermenter because additional problems can be diagnosed by up scaling of newly developed strain. This process is strenuous but needs to be executed because results can differ extensively from lab scale process. The range of inconsistency can vary from strain to strain but can't be anticipated until definite pilot scale fermentation is carried out. This variation is due to the difference in stirring and aeration of both lab scale and pilot scale fermentation Reversion of newly developed highly efficient strain to a low performance strain is a common problem associated with this process. This can happen during the process of industrial fermentation due to the loss of production capacity or phenotype alteration. Thus this strategy is an essential step for final development of a high performance strain (Lee & Kim, 2015).

3. Techniques for strain improvement

3.1 Mutation

Mutagenesis is the main reason behind genetic variation but it is not possible for a single mutagenic treatment to give us all possible kinds of mutations required. Mutagen (agent that causes mutation) can be distinguished into two categories: physical mutagens (ultraviolet, gamma and X-rays) and chemical mutagens (ethyl methane sulphonate - EMS, nitrosomethyl guanidine – NTG, etc.). The type of mutation produced is determined by the kind of DNA damage induced by mutagen as well as the influence of DNA repair pathways on this damage. The mutagen inflicts various sorts of damage to the DNA molecule but the degree to which this DNA damage is caused depends on the type of mutagen used

(Rowlands, 1984). For example, UV radiation gives huge percentage of pyrimidine dimers, ionising radiation causes chromosomal distortion at a very high rate whereas EMS and NTG cause alkylation (Auerbach, 1976).The enzyme mechanism involved chooses the DNA repair pathways i.e. either mutagenic or non-mutagenic. The non-mutagenic pathways include photoreactivation, excision repair and recombination repair. However the best understood mutagenic pathway is SOS repair in Escherichia coli. This pathway is in known to be associated with the repair of DNA damage caused by both physical and chemical mutagens (Kenyon, 1983; Witkin, 1976).

Another circumstance which should be taken into account when selecting a mutagen is its specificity. Although the concept of mutagen specificity is not clearly understood but we know the mutagen acts on certain parts of the genome while rarely affecting others, if at all (Auerbach, 1976). The industrial geneticist is unable to anticipate the class of mutation required to improve the strain. Therefore, it is advised to try out several types of mutagens to create a wide collection of mutants which can then be further screened for efficiency (Rowlands, 1984).

The optimum dose of any mutagen is referred to as the amount that results in maximum percentage of required mutants in population of cells that survive. The trace of mutant cells per survivor plotted against increasing dose is more relevant than number of mutants against dose. Any mutation plot gives a curve almost identical to curve A or curve B, as shown in Figure 2, and it doesn't depend on the type of mutagen used rather on the phenotype of the mutation. If there is improvement in product titre and yield we will get a curve similar to the curve B. High doses of mutagen decrease the chances of survivors and can also result in undesired secondary mutations. Therefore, the optimisation of mutagen dose is essential (Rowlands, 1984).

3.1.1 Spontaneous mutations

The naturally occurring mutations in the cell are called spontaneous mutations. These mutations occur because of the following reasons (1) mispairing errors (2) depurination (3) deletions and insertion sequences (4) error-prone DNA repair mechanisms. Spontaneous mutations have low frequency of occurrence and usually occur at about 10^{-10} to 10^{-6} per generation per gene. Selection pressure can be used to screen and isolate spontaneous mutants. These mutants can then be further subjected to physical or chemical mutagens to

develop an industrial strain (Saxena, 2015). A good example of this is the spontaneous mutation of wild as well as mutant strains of Penicillium chrysogenum to improve the production of its metabolite (Kardos & Demain, 2011).

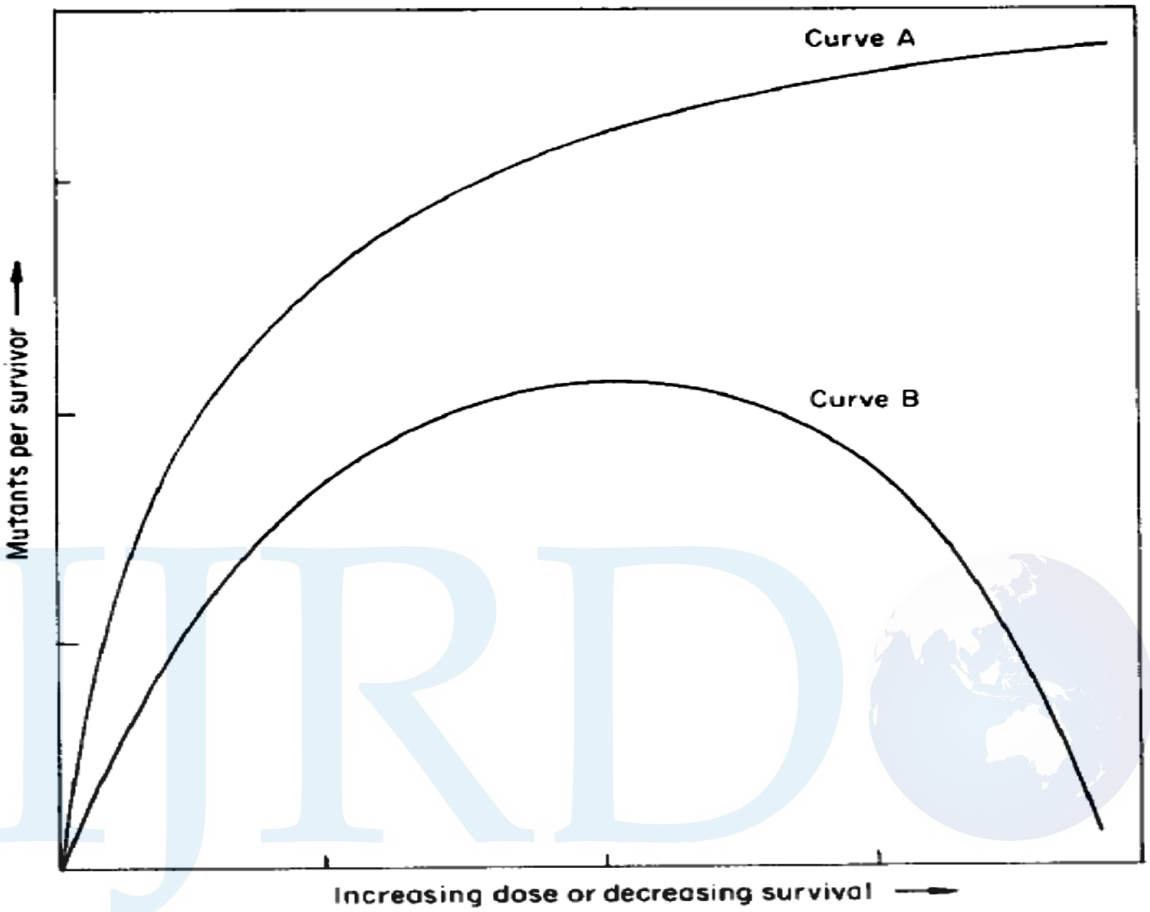

Figure 2. Typical mutation induction kinetics curve

3.1.2 Classical mutagenesis

This type of mutagenesis involves the usage of physical and chemical mutagenic agents to manipulate the genetic structure of microbes. This is done to improve the desired characteristic of an organism. The procedure of mutagenesis involves following steps: (1) exposure of parent strain to a mutagen (2) random screening of survivors (3) assay of fermentation medium for enhanced formation of product. Every time an improved strain is obtained it is utilised as a parent strain for in next cycle and this process continues until a strain with high throughput is developed (Saxena, 2015).

3.1.3 Directed mutagenesis

The mutations discussed above are totally random and there is no way of knowing the site a certain mutagen is going to affect. In cases, where the gene responsible for the function, that needs improvement, is known we can use site directed mutagenesis. A prerequisite for directed mutagenesis is the knowledge of genes to mutate and the availability of tools for directing the mutagenesis to specific genes controlling the product formation. It has been reported that actively transcribing genes are more susceptible to mutagenesis, so mutagenesis during the period of highest biosynthetic activity will result in improved product titre mutants. This is carried out optimally in a continuous culture fermenter because the conditions of maximum biosynthetic activity can be judged (Rowlands, 1984).

Another approach for directed mutagenesis is the use of NTG because of its specificity to mutate at the replication fork. Mutagenesis can be set at the specific site by setting up a proper time dependant mutational map (Rowlands, 1984). A third method that can be adopted is in vitro mutagenesis, which is dependent on recombinant DNA and cloned genes availability. This permits the isolated genes or parts of the genes such as promoters to be specifically mutated in the test tube (Shortle, Dimaio & Nathans, 1981; Timmis, 1981).

3.2 Recombination

Mutagenesis and recombination are the dominant techniques for strain improvement as compared to recombination. Recombination is more successful if it is used as a complement and not as an alternate technique. Recombination is the process in which two genetically different strains combine to generate a hybrid that is superior and different from either of the parents. Recombination is useful in erasing the neutral and deleterious which arise during random mutagenesis (Saxena, 2015).

First homologous recombination method, DNA shuffling technique was established by Stemmer in 1994. This technique digests the genes randomly into short fragments which are then reassembled into full length fragments by PCR. This technique was shown to generate strains with improved phenotypic as well as production characterisctics (Stemmer, 1994). Figure 3 shows some of the techniques for recombination (Saxena, 2015). The major advantage of this technique is that the developed strains are not considered genetically modified microorganisms (GMMO).

3.3 Recombinant DNA technology

Advances in molecular biology techniques have opened up new doors for strain improvement. Recombination DNA technique has initially been used to improve titres of primary metabolites such as amino acids and extracellular enzymes (Gouka, Punt, & van den Hondel, 1997). Recombination technique involves cloning of genes and expression of cloned gene into an expression vector and its product formation. The expression vector increases the amount of product produced in the presence of an inducer such as: IPTG.

Category of improvement	Microorganisms	Results	Reference
Substrate uptake	*Lactobacillus delbrueckii* and *Bacillus amyloliquefaciens*	Non-fastidious strain obtained after three rounds of genome shuffling for direct conversion of starch to lactic acid	John et al. (2008)
	Sphingobium chlorophenolicum	Higher tolerance level of pentachlorophenol for degradation at a faster rate	Dai and Copley (2004)
Strain tolerance to end product	*L. rhamnosus*	More accumulation of lactic acid at 3.6 pH compared to wild type	Wang et al. (2007)
	Streptomyces pristinaespiralis	Four rounds of protoplast fusion generated 100-μg/ml pristinamycin resistant recombinant	Xu et al. (2008)
	Saccharomyces cerevisiae	Improved strain maintaining cell viability till 55 °C and 25 % ethanol stress after three rounds of genome shuffling	Shi et al. (2009)
Product yield enhancement	*Streptomyces fradiae*	Two rounds of genome shuffling led to six times higher titre of tylosin	Zhang et al. (2002)
	Streptomyces gilvosporeus	High natamycin-producing strain – approximately 153 % higher than the parent strains and 1.17 times more than present strain	Zhu et al. (2006)
	Phaffia rhodozyma	Two cycles of recursive protoplast fusion, a shuffled strain was selected and 1.43 times higher yield of astaxanthin was obtained	Zheng and Zhao (2008)
	Sorangium cellulosum	The epothiolone production of the fusant was increased about 130 times compared to the starting strain by 3 rounds of genome shuffling	Gong et al. (2009)
	Clostridium diolis DSM 15410	Improvement of 1,3-propanediol production by 4 rounds of genome shuffling by 80 %	Otte et al. (2009)
	Propionibacterium shermanii	Enhanced vitamin B_{12} production after genome shuffling	Zhang et al. (2010)

Figure 3. Techniques involving recombination for strain improvement

3.4 Integrated strain improvement: Precision engineering technology

Precision engineering technology or integrated strain improvement is a newly developed aspect that also involves strain improvement. Previous approaches to strain improvement ignored the negative effects on the industrial microbes such as; formation of undesired products or slow growth, substrate specificity and weak stress tolerance. Therefore, the industrial geneticists are shifting towards the metabolic engineering approaches. Precision engineering technology involves the integration of classical metabolic engineering and screening methods with profiling technologies which give a more clear understanding of genetics and physiology of metabolite production. The component parts of precision engineering technology are shown in Figure 4 (Saxena, 2015). Precision engineering technology is still in need of better understanding and development before its complete potential can be realised. Precision engineering is a good framework of old and new techniques and is going to have a huge impact on industrial biotechnology.

4. Screening of improved strains

4.1 Random Screening

4.1.1 Surface culture screens

Surface culture screens can be performed in two ways: the first one is based on the diffusion of product from the colony forming a zone which can then be measured. The second screening method is based on the elution of product from growth medium which can then be assayed. Zone assays can be carried out by overflowing the colonies with an assay mixture, which can be either biological or chemical. A bioassay mixture consists of a sensitive test organism, whereas a chemical assay medium consists of a chromogenic substance which reacts with the product and gives a colour reaction. The problem associated with this type of screen is that the relationship between product titre and zone diameter is decreasing exponential relationship i.e. a huge increase in titre results in decrease in zone diameter. To increase the resolution of this method zone area can be measured instead of zone diameter Elution assays require considerable time and effort but have an advantage that once the product is in the solution various quantitative tests can be performed (Rowlands, 1984).

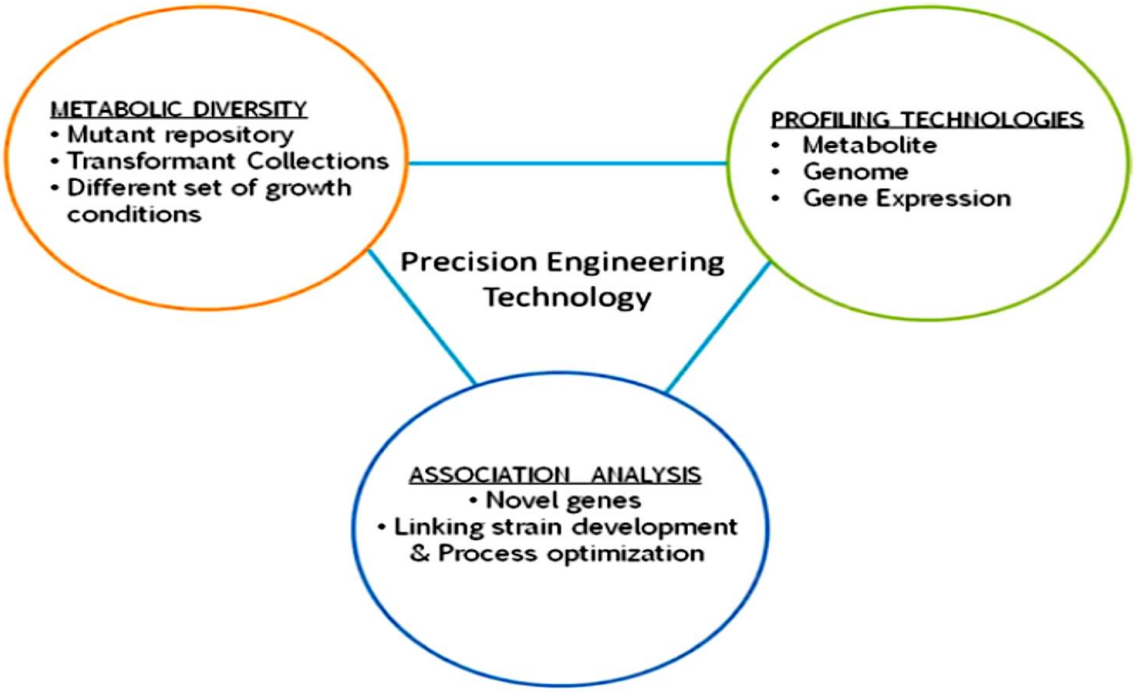

Figure 4. Components of precision engineering technology for strain improvement

4.1.2 Liquid culture screens

These screens are based on Erlenmeyer shake flasks. Liquid culture screens are advantageous over surface culture screens because they can mimic production conditions better. Aeration can be controlled in liquid culture screens. Generally this screening method requires time and attention so few isolates per unit time are tested. These are therefore used for high resolution screens (Rowlands, 1984).

4.1.3 Multi-level screening

When the testing error of screen is high, multi-level screening is the best option. In multi-level screening the isolates pass through different stages of screening where the top best strains are selected to go through the next stage. The early stages of multi-level screening have low resolution but the resolution increases as the screening proceeds. The purpose of early stage is to remove the strains that give low titre of product. It is important to ensure that there is no antagonistic reaction between different types of strains used and the selection pressure used should be common to all levels of screening (Rowlands, 1984).

4.2 Rational screening

Table 2. Different method for selective screening of mutants

Method	Description	Reference
Phenotypic titre depression	Inhibitors of product biosynthesis are added to the screening medium – improved strains overcome this metabolic block by overproducing metabolites	(Rowlands, 1984)
Use of auxotrophs	Titre production inhibited genetically by auxotrophic mutations in primary metabolite pathway which supply precursor for desired product – improved titre can be selected.	
Reversion of non-producers	Genetic suppression of titre decreasing mutations – generates strains with genuine titre increase	
Selective detoxification	Growth inhibitory toxic chemicals are added that react with the product of interest	
Resistance/Sensitivity to toxic analogues	Resistance of the products to toxic analogues is checked	

5. Conclusion

There are a wide range of techniques that can be utilised with proper strategy to improve a microbial strain for overproducing industrial products. The metabolic engineering literature is filled with data describing how to improve the microbial strain but very few strains have actually been scaled up at industrial level because proper channels between industrial and

academic level don't exist. This bridge needs to be established which will ultimately result in tremendous new opportunities for industrial biotechnology.

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