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PRODUCTION OF MICROBIAL METABOLITES AND OPTIMIZATION OF KEY FACTORS INVOLVING THEIR HYPERPRODUCTION IN BATCH CULTURE (REVIEW)

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ABSTRACT

Fermentation is a metabolic process which uses the sugar in the absence of the Oxygen. However, it can be done both aerobically and anerobically. The products obtained from this process include organic acid, alcohol, gases and many others by-products. One such industrially important by-product is metabolite production. Metabolites are small molecular weight compounds (<2,500 amu) produced as an intermediate or end product of a metabolic reaction. Microorganisms during fermentation process produce not only primary metabolites but secondary, tertiary and quaternary metabolites as well. These metabolites are industrially very important, however for industrial applications the wild-type strains of micro-organisms need to be modifying by strain improvement techniques to enhance the productivity of commercially important compounds. This review will focus on the production of primary and secondary metabolites production with emphasis to the use of both liquid and solid state fermentation along with the various approaches for the enhanced production of the metabolites. Factors such as pH, temperature, types of nutrients, carbon and nitrogen sources, growth factors, metabolic regulators, effect of water, presence of other microbes, strain used and other parameters are also discussed in the view of hyper-production and optimization of the metabolites.

KEYWORDS: Primary metabolites, Fermentation, Trophophase, Vitamins, Secondary metabolites.

INTRODUCTION

Metabolites are small molecular weight compounds (<2,500 amu) produced as an intermediate or end product of a metabolic reaction.[1] Microorganisms produced two main types of metabolites such as primary and secondary metabolites. These metabolites are produced during different phases of their growth. Primary metabolites are produced during the log phase or trophophase of growth and are essential for the development of microorganism. These metabolites include amino acids, proteins, nucleotides, nucleic acids, carbohydrates and lipids.[2] Various primary metabolites are of industrial importance and produced by fermentation at large scale as described in Table 1. Metabolites produced by wild-type microorganism only fulfill the requirements of the producing organism. But for industrial applications this wild-type needs to be modifying by strain improvement techniques to enhance the productivity of commercially important compounds.[3]

Secondary metabolites are produced during the stationary phase also called the trophophase. These metabolites are not required for the growth but have various industrial applications. Secondary metabolism is the property of slow-growing microorganisms. Both primary and secondary metabolisms are interrelated as illustrated in Fig 1. Primary metabolites are produced by majority of microorganism but secondary metabolites are produced only by the filamentous bacteria and fungi as well as by some sporing bacteria.[2]

Secondary metabolites include antibiotic agents, enzyme inhibitors, growth promoters and other pharmacologically important agents. These products are the basis of various fermentation processes.

Table 1: Commercial applications of primary metabolites.

Primary metabolite	Application
Amino acids L-glutamic acid L-lysine L-threonine L-isoleucine	Flavor enhancer Growth enhancer and feed supplement Used in the feeds of pig and poultry Feed additive
Nucleosides and nucleotides	Flavor enhancer
Vitamins	Feed supplements
Organic acids Lactic acid Citric acid Acetic acid	Various uses in the food industry
Ethanol	'Active ingredient' in alcoholic beverages. Used as a motor-car fuel when blended with petroleum
Phenyalanine	Precursors of aspartame, sweetener
Polysaccharides	Applications in the food industry Enhanced oil recovery
Glycerol	Manufacture of drugs, food, cosmetics, paint and many other commodities
1, 3-propanediol	Used as a lubricant and solvent
Erythritol	Non-carcinogenic, non-caloric, and diabetic-safe sweetner

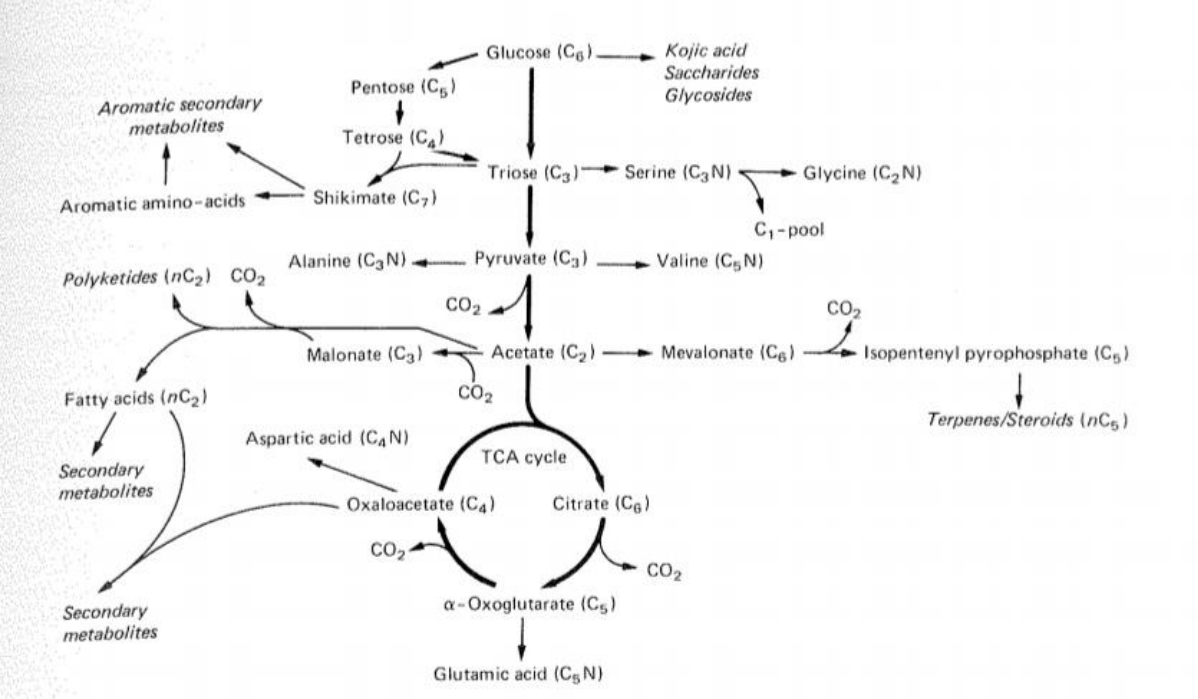

Figure 1: The interrelationship between primary and secondary metabolism. Primary catabolic routes are shown in heavy lines and secondary products are italicized.

Secondary metabolites

Biosynthetic families

Microorganism produced chemically diversified secondary metabolites. However, their biosynthetic pathways are linked to the primary metabolism. Following are the biosynthetic categories of SM:

(1) Secondary metabolites derived from aromatic amino acids (shikimic acid). For example, "ergot alkaloids" and the antibiotics chloramphenicol and candicin.

(2) Metabolites derived from amino acids such as B-lactam antibiotics (cephalosporins, penicillin and cephamycins) and cyclic peptide antibiotics (gramicidine and immunosuppressive agents)

(3) Secondary metabolites obtained from Acetyl-CoA. This family of metabolites can be further subdivided into "polyketides" and "terpenes". Polyketides include the antibiotic (erythromycin), the insecticidal-antiparasitic compound (avermectin) and the antitumour agent (doxorubicin). An example of the terpenes is the non-cytotoxic antitumor agent (taxol).

(4) Sugar derived metabolites such as streptomycin and kanamycin.[4]

Since, the routes of biosynthesis of SM are related to the primary metabolites, therefore both types of metabolites use the same regulatory mechanisms for control such as induction, carbon source regulation and feedback regulation.

Production methods

Liquid fermentation

Submerged fermentation (SmF) by using batch or fed-batch culture techniques is the general method for the production of SM at industrial level. An inoculum of improved strain is prepared in the flasks containing medium and then transferred to the "seed culture" or small fermenter. The culture, when in exponential growth phase, is transferred to the production fermenter with the range of 30,000 to 200000 liters. Parameters like, temperature, pH, medium composition, aeration rate and agitation, are optimized. Environmental conditions are manipulated for different regulatory mechanisms.[5] For cephalosporine fermentation, methionine as an inducer is added to the medium. Similarly, for the fermentation of chlortetracycline phosphate is restricted and in case of penicillin production glucose is limited. Antibiotic fermentation processes are regulated by utilizing carbon sources such as lactose. Soybean meal is used as a nitrogen source to avoid regulation of nitrogen. Sometimes, concentration of a metabolite is increased by the addition a particular precursor for example in case of cephamycin production by *Streptomyces calvuligerus* lysine is added as a cofactor and precursor.[6] Special turbine impellers are used for agitation and air supplied at rates of 0.5-1.0 v/v per min. concentrations of O_2 and CO_2 are analyzed by the exit gas. In the subsequent stages some secondary metabolites are chemically modified to synthesize semi-synthetic derivatives.[5]

Figure 2: A setup of Submerged Fermentation for the production of secondary metabolites.

Solid-state fermentation

Solid-state fermentation (SSF) is potentially more important for the production of secondary metabolites.[7,8,9] According to modern definition "SSF is a microbial culture that develops on the surface and at the interior of a solid matrix and in absence of free water". SSF is divided into two types that depend on the nature of solid phase.[10]

(1) Solid-culture of one support-substrate phase—the solid phase consists of a material that acts as both support and nutrient source. Animal goods and waste are used as solid phase

(2) Solid culture of two substrate-support phase—the solid phase consists of an inert support impregnated with a liquid medium. Inert support act as the source of nutrient and water. Sugarcane bagasse pith or polyurethane can be utilized as inert support.

SSF is suitable for actinomycetes and fungi, because the conditions are suitable for the growth of these microorganisms. Some of the metabolites produced by SSF are given in (Table 2).

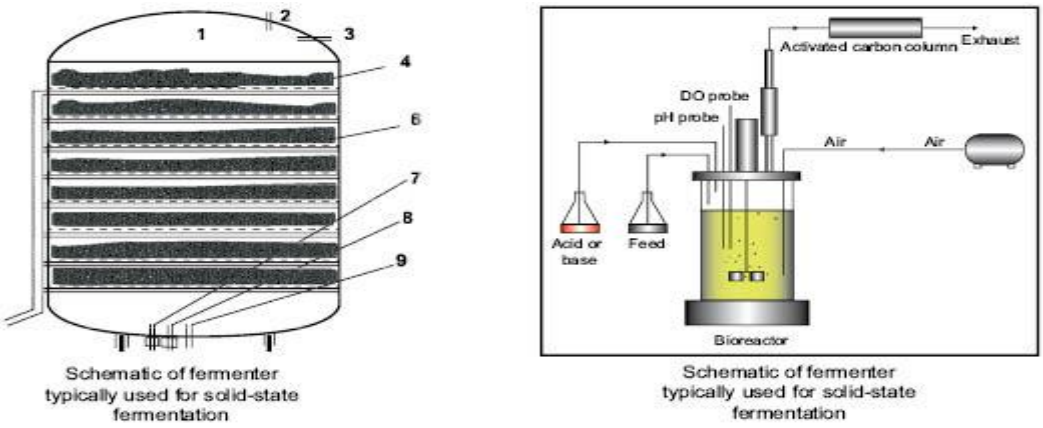

Figure 3: A Schematic representation of Solid State Fermentation.

Table 2: Biological activities of some microbial secondary metabolites of industrial importance.

Activity	Examples	Producing Microorganism
Antibacterials	Cephalosporin	*Acremonium chrysogenum*
	Cephamycin	*Streptomyces clavuligerus*
	Chloramphenicol	*Streptomyces venezuelae*
	Erythromycin	*Saccharopolyspora erythraea*
	Kenamycin	*Streptomyces kanamyceticus*
	Tetracyclin	*Streptomyces aureofaciens*
	Penicillin	*Penicillium chrysogenum*
	Rifamycin	*Amycolatopsis mediterranei*
	Spectinomycin	*Streptomyces spectablis*
	Streptomycin	*Streptomyces griseus*
Anticholestrolemics	Lovastatin	*Aspergillus terreus*
	Monacolin	*Monascus ruber*
	Pravastatin	*Penicillium citrinum*
Antifungals	Amphotericin	*Streptomyces nodosus*
	Aspergillic acid	*Aspergillus flavus*
	Aureofacin	*Streptomyces aureofaciens*
	Candicidin	*Streptomyces griseus*
	Griseofulvin	*Penicillium griseofulvum*
	Nystatin	*Streptomyces nourse*
	Oligomycin	*Streptomyces diastachromogenes*
Antitumorals	Actinomycin D	*Streptomyces antibioticus*
	Bleomycin	*Streptomyces verticillus*
	Doxorubicin	*Streptomyces peucetius*
	Mitomycin	*Streptomyces lavendulae*
	Taxol	*Taxomyces andreanae*
Enzyme inhibitors	Clavulanic acid	*Streptomyces clavuligerus*
Plants Growth Regulators	Gibberellin	*Gibberella fujikuroi*
Growth Promoters	Monensin	*Streptomyces cinnamonensis*
	Tylosin	*Streptomyces fradiae*
Herbicidals	Bialaphos	*Streptomyces hygroscopicus*
Immunosuppresives	Cyclosporin A	*Tolypoclaudium inflatum*
	Rapamycin	*Streptomyces hygroscopicus*
	Tacrolimus (FK-506)	*Several Streptomyces species*
Insecticides Antiparasitics	Avermectin	*Streptomyces avermitilis*
	Milbemycin	*Streptomyces hygroscopicus*
Pigments	Astaxanthin	*Phaffia rhodozyma*
	Monascin	*Monascus purpureus*

Factors effecting production of metabolites

Effect of pH

pH is actually the measure of the acidity and alkalinity of a solution. pH has a great affect in the fermentation process for metabolite productioneven a slight change in the pH can cause the toxicity or cease the reaction, therefore pH is needed to be optimized.[11] For the production of ethanol that is a commercially important

primary metabolite the enzymes only work best in the acidic conditions. This is due to the acidic condition provided by the pyruvic acid. Most of the fermentation processes if the pH drops below 4.2 and therefore the optimum pH for fermentation is about 4.8 - 5.0.[12] But again each strain i.e bacterial or yeast used for the fermentation has their own optimum pH.

The pH will not only affect the strains and species used for the fermentation process but will also affect their metabolic activities as well. Different strains have different optimum pH, for instance the *Oenococcus* can tolerate low pH easily as compare to the *lactobacilli* or *pediococci.*

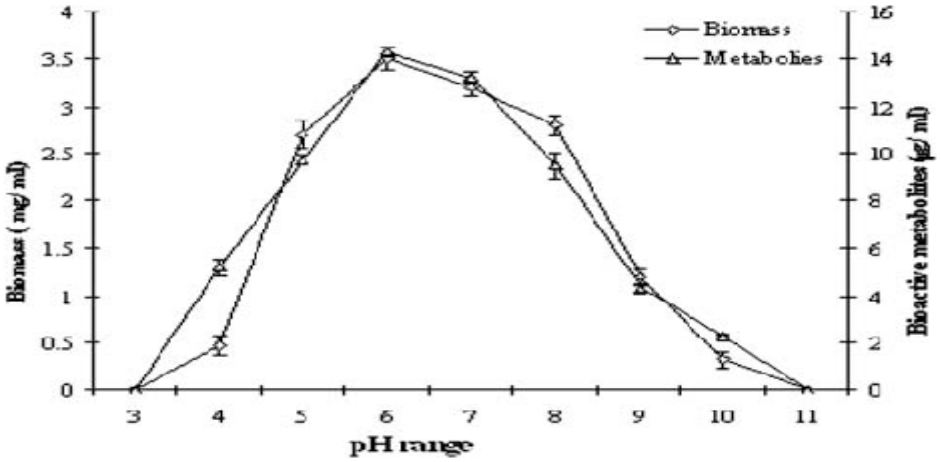

Figure 4: Effect of pH on metabolite production.

Effect of temperature

Temperature changes have an intense effect on the living things. Enzyme-catalyzed reactions are particularly sensitive to the small changes in the temperature. The temperature can affect the process of fermentation in the following ways: (1) Effect of high temperature (2) Effect of Low temperature (3) Optimal Temperature (4) Control of Temperature (5) Temperature and pH.

For the production of secondary metabolites total biomass production should be low that could be achieved either at very low or high temperature because at this temperature enzymatic function is ceased that effect both the production of primary metabolites and biomass. For the production of wine the organisms will be able to grow rapidly at high temperature but it'll affect the quality of wine and deteriorate it. In case of the yeast, low temperature is preferred. However, the rate of fermentation should increase with the increase in temperature from the 10°C to 40°C while at 50°C and temperature above than this the rate of the fermentation will decrease.

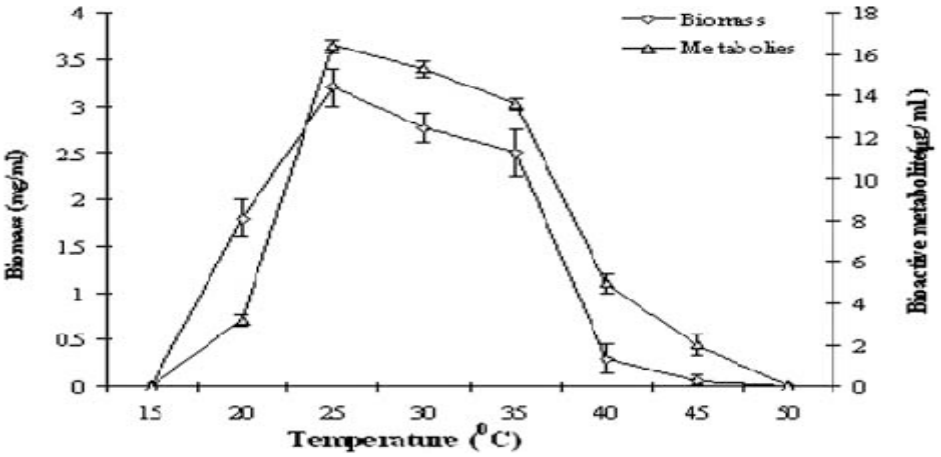

Figure 5: Effect of temperature on the production of metabolites.

Types of the Nutrients and their effects

Simple Nutrients

There are six basic nutrients needed which includes Carbohydrate, proteins, fats, vitamins, water and minerals for the fermentation process. Their sources will be explained later.

Complex Nutrients

There are few media which are complex and are needed in the fermentation. They are chemically synthesized and are specially provided in the fermentation process. Just as an example of the lactic acid bacteria, which require the compound 4'-O-(β-D-glucopyranosyl)-D-pantothenic

acid which is found in tomato juice and in other fruit juices. It is actually a growth factor needed by the lactic acid bacteria.[13] Therefore the media used for the cultivation of the lactic acid bacteria usually contain apple or tomato juice as essential components. If the yeast is present in the media it will interfere with the production of the lactic acid and may deplete it but if the lysis of the yeast[14] occur as in wine making than the lactic acid will again be activated however if the nutritional content is low than the growth of lactic acid may slow down. Other complex media also contain the high juice solids content and the extended skin contact which increase the nutritional content for the lactic acid bacteria and increase its growth as well.[15]

Effect of water in the Fermentation process

Water is one of the important components in the fermentation process and is used in the fermentation for many processes like the cooling, heating and rinsing. Therefore clean and a continuous supply of water is needed in order to maintain the salt dissolution, pH and other effluent contamination.

The mineral content of the water is quite important in the process of brewing. And it is very critical in the process of mashing. These days, water is also been treated with the deionization or many other techniques, additives like salts are also added and pH is also varied to get the required product and different flavors of beer.

Reuse of water is also an important factor affecting the fermentation process.[16,17]

Carbon Source and its effect

The rate at which carbon source is utilized can affect the formation of the biomass or the other products like primary and secondary metabolites obtained from the fermentation process. If the sugars are being highly utilized then it will lead to the low productivity of the secondary metabolites[18] (Table 3). Therefore, alternative methods are opted for the producer organism to give the high yield.

Table 3: Carbon catabolite regulation of metabolite biosynthesis.

Metabolite	Microorganism	Interfering carbon source	Reference
Griseofulvin	*Penicillium griseofulvin*	Glucose	Rhodes *et al*[19]
Penicillin	*P.chrysogenum*	Glucose	Pirt and Rhigelato[20]
Cephalosporin	*Cephalosporium acremonium*	Glucose	Matsumura et al[21]
Aurantin	*Bacillus aurantinus*	Glycerol	Nishikiori et al[22]
a-amylase	*B. licheniformis*	Glucose	Priest and sharp[23]
Bacitracin	*B. licheniformis*	Glucose	Weinberg[24]
Puromycin	*Strptomyces alboniger*	Glucose	Sankaran and Pogell[25]
Actinomycin	*S. antibioticus*	Glucose	Marshell et al[26]
Cephamycin C	*S. clavuligerus*	Glycerol	Aharonowitz[27]
Neomycin	*S. fradiae*	Glucose	Majumdar[28]
Cycloserine	*S. graphalus*	Glycerol	Svensson[29]
Streptomycin	*S.griseus*	Glucose	Inamine et al[30]
Kanamycin	*S. kenamyceticus*	Glucose	Basek and Majumdar[31]
Novobiocin	*S. niveus*	Citrate	Kominek[32]

Nitrogen Sources

Most of the industrial micro-organisms either use organic or inorganic nitrogen sources for fermentation Ammonia gas has been used as an inorganic source, which is not only used in the define medium but also used to maintain the pH. Apart from ammonia, ammonium salts and nitrates are also being used.[33]

According to a research the production of antibiotics by various microorganisms depends on the type and the concentration of nitrogen source.[34] Rapidly metabolizing nitrogen sources such as ammonium ion (NH_4^+), nitrate ion and various amino acids can be inhibitory for the production of antibiotics. In a culture medium the production of antibiotics only starts when the entire nitrogen source has been utilized.

Consumption of complex nitrogen sources for the production of antibiotic helps to establish the physiological conditions in trophophase that favors the enhanced production of antibiotic in the idiophase.[35] For the production of polyene antibiotics, the soybean meal has been considered the most favorable source due the balance of nutrients, low ratio of phosphorus and the low rate of hydrolysis. The process of slow hydrolysis prevents the piling up of ammonium ions and inhibits the "repressive amino acids". Therefore, selection of an ideal nitrogen source is necessary for the production of secondary metabolites. (Table).[36] Mostly the use of complex nitrogen sources is avoided due to the downstreaming and the effluent treatment problems.

Table 4: Best nitrogen sources for some secondary metabolites.

Product	Main nitrogen source	Reference
Penicillin	Corn-steep liquor	Moyer and Coghill[37]
Bacitracin	Peanut granules	Inskeep et al[38]
Riboflavin	Pancreatic digest	Malzahn et al[39]
Novobiocin	Distillers solubles	Hoeksema and Smith[40]
Rifamycin	Pharmamedia	Sensi and Thiemann[41]
Gibberellins	Soybean meal, Ammonium salt and natural plant nitrogen source	Jefferys[34]
Butirosin	Dried beef blood or haemoglobin	Claridge at al[42]
Polyenes	Soybean meal	Martin and MacDaniel[33]

Minerals and their effect

In the fermentation the micro-organisms need the minerals to complete the process. The minerals which are normally needed by the micro-organisms include magnesium, calcium, phosphorous, sulphur, potassium, and chlorine are the essential minerals needed by the micro-organisms. Some other minerals are copper, cobalt, manganese, iron, zinc and molybdenum.[43] They are also essential but the impurities introduced by them not only cause the toxicity in the fermentation media but also affect the quality and yield of product of the fermentation.[44] Some of the minerals like phosphate are added in the media to act as a buffer which would help maintain the pH of the fermentation process.

For the production of secondary metabolites choice and concentration of some minerals is very critical because they have low tolerance range for various inorganic phosphates. According to Gray *et al*.,[45] the production of streptomycin can be improved by precipitating out the excessive inorganic phosphates by using calcium ions. Liras *et al*.,[46] reported that phosphate also have the ability to inhibit or repress the enzymes of a metabolic pathway. Sometimes, chlorine is used to supress the formation of non-chloro compounds. For example in the production of griseofulvin about 0.1% KCl is used to fulfil the requirement of chlorine.[47]

Growth factors and their effects

Some micro-organisms are not able to synthesis all the components therefore they need components and these components are called the growth factors. The most common growth factors are vitamins, fatty acids, amino acids and sterols.[48] Micro-organisms may either utilize all of these factors or they need few of them to grow properly. Most of the nitrogen and carbon sources however provide all the necessary components for the growth of the microbe. However, if the vitamin deficiency occurred then it is mostly eliminated by the careful blending. It should be noted that if only one vitamin is needed by the fermentation process than it is recommended to use the pre vitamin itself rather than a mixture example thiamine and glutamic acid etc.

Thus, the depletion of any of the nutrients, their excess or their toxicity will highly affect the fermentation process.

Approaches to improve the yield of secondary metabolites

Strain improvement

Strain improvement is a science or technology used to manipulate the microbial strains to enhance their metabolic capacities for biotechnological applications. Increased production of desired metabolite is the main purpose of strain improvement.[49] These days, two strategies can be alternatively employed for the development of improved strain: (1) Classical genetic methods (2) molecular genetic methods.

Classical genetic methods

In these methods, mutation of particular strain is followed by random screening. Further, fermentation tests are performed to select the best improved strain. Physical mutagens such as ethyl methanesulphonate and nitrosoguanidine can be used for the induction of mutation.[50] Main advantages of mutation are its simplicity, requirement of sophisticated equipment and its effectiveness. Development of these methods requires the basic knowledge of product metabolism and the pathway through which it is produced. Random screening methods remove all the undesired genotypes and only select the particular desired one strain.

Microorganism possesses regulatory mechanisms that prevent the overproduction of a particular metabolite. However, it has been reported that through mutation these mechanisms can be eliminate or decreased to favor the overproduction of a primary metabolite. But for secondary metabolites the nutritional conditions of culture are manipulated because the control mechanisms for secondary metabolite production are more complex.[51] Genetic recombination methods are also important complement to mutagenesis. In these methods a strain is constructed that has combinations of mutations. Recombination through protoplast fusion is one the most common method used for strain improvement. In this method a strain that is already improved by genetic methods is fused with a new isolate to enhance its production capacities.[52]

Molecular genetic methods

Adequate vectors, identification of the biosynthetic pathway and effective transformation protocols are required for the application of molecular genetic methods. After this, biosynthetic genes are cloned and

analyzed. Following are some strategies used for strain improvement:

(1) Characterization of high producing strains. By employing genetic engineering, genes responsible for antibiotic synthesis are grouped together in "fungi" and "*actinomycetes".* For example, in penicillin producing strains of *penicillum chrysogenum* AS-P-78 the cluster of biosynthetic genes has been amplified.[53]

(2) Targeted amplification of secondary metabolite producing strains by using two different approaches such as amplification of targeted gene and amplification of whole pathway.

(3) Inactivation of competing pathway. By using these methods a pathway competing for metabolites can be blocked. This can be achieved by gene disruption, insertion of an antisense synthetic gene and by transposon mutagenesis.[54]

(4) An alternative of blocking method is the amplification of a regulatory gene.

(5) Strain improvement can also be done by the introduction of a heterologous gene. Expression of heterologous gene leads to the production of a new product that is industrially important. But this process is chemically difficult and expensive.[55]

(6) In combinatorial biosynthesis, non-conventional compounds are used as substrate for the production of a new antibiotic.

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