**Metabolic Engineering**
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**Lecture-37**
**Applications of Metabolic Engineering in Amino Acids Production**

In today's lecture we will be discussing about the application of metabolic engineering in amino acid production.

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The concepts those will be covered in this lecture include applications of metabolic engineering for amino acid production particularly in the food industry and pharmaceutical industry, history and advancement of microbial production of amino acids, strategies for systems metabolic engineering of microorganisms for the production of amino acids and case studies of systems metabolic engineering in *Corynebacterium glutamicum*.

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Metabolic engineering for amino acid production.

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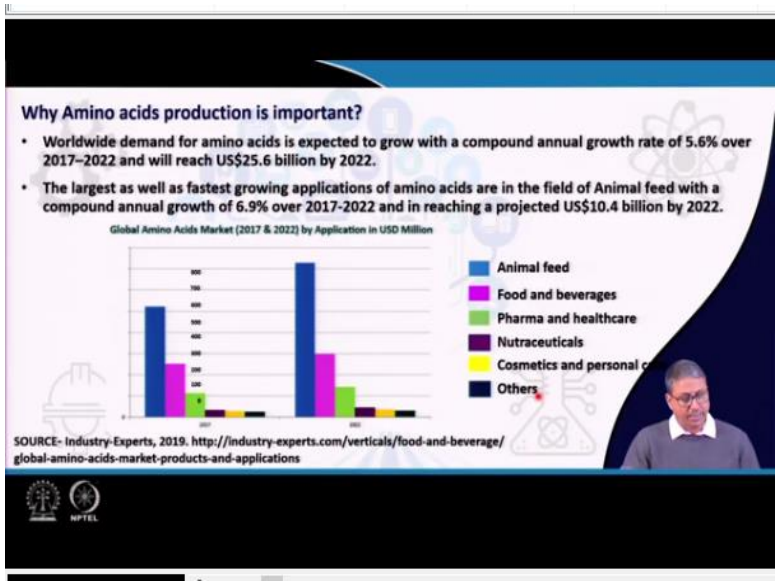


Why amino acids are important? Amino acid productions are important because of many industrial reasons and worldwide demand for the amino acid is expected to grow with a compound annual growth rate of 5.6% over 2017 to 2022 and it is expected to reach US dollar 25.6 billion by 2022. The largest as well as the fastest growing application of amino acids are in the field of animal feed with a compound annual growth of 6.9% over 2017 to 2020 and reaching a projected US dollar 10.4 billion by 2022.

So, here are the some of the statistics available on different applications in animal feed, food and beverage, pharma and health care, nutraceuticals, cosmetics and personal care and others.

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Now these applications of amino acids are broadly categorized into 2 different major areas one is the food industry, another is the pharmaceutical industry. So, within the food industry preservatives, flavour enhancers and increasing nutritional values are the major requirements. As preservatives aspartame, a dipeptide compound is used as a low calorie artificial sweetener in soft-drink industry.

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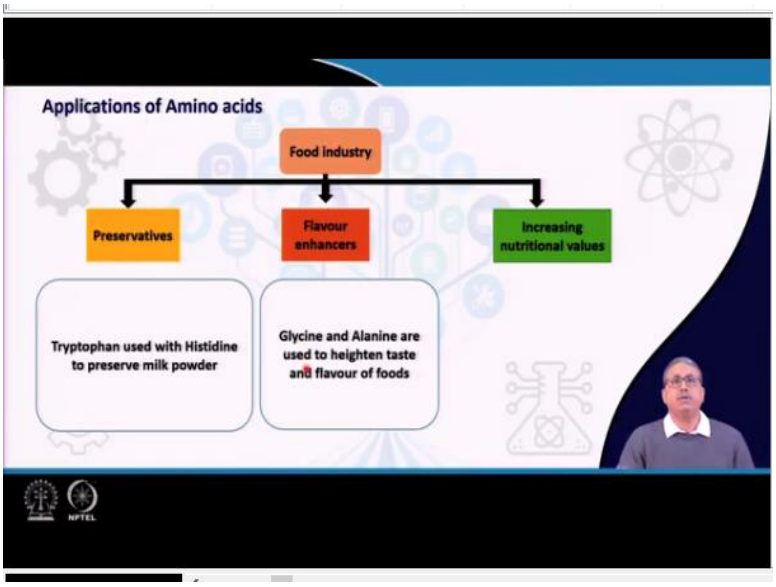


Tryptophan is used with histidine to preserve the milk powder. Glycine and alanine are used to heighten the taste and flavour of the foods.

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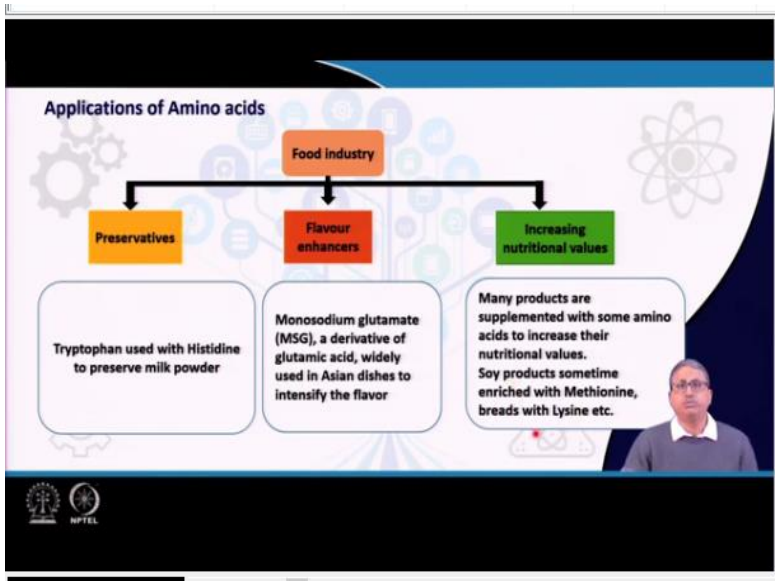


While monosodium glutamate is a derivative of glutamic acid is widely used in Asian dishes to increase the flavour and many products supplemented with some amino acids to increase their nutritional values including the soya products, sometimes enriched with methionine or the breads with lysine.

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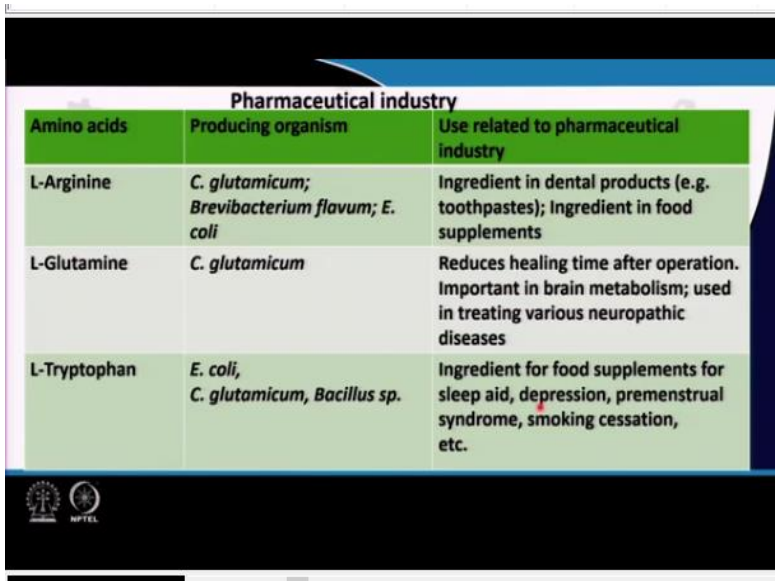


Now within the pharmaceutical industry amino acids, arginine, glutamine and tryptophan are mostly applied and these are produced through different high yielding strains which are improved through metabolic engineering including *Corynebacterium glutamicum*, *Brevibacterium flavum*, *Escherichia coli*, *Bacillus* species and these amino acids are used as ingredients of different medical purposes including the dental products, including the medicines which are used to heal the operated tissues important in brain metabolism and treating various neuropathic diseases.

These are also used as ingredients for food supplements to help sleeping, sleep, depression, premenstrual syndrome, smoking cessation etcetera.

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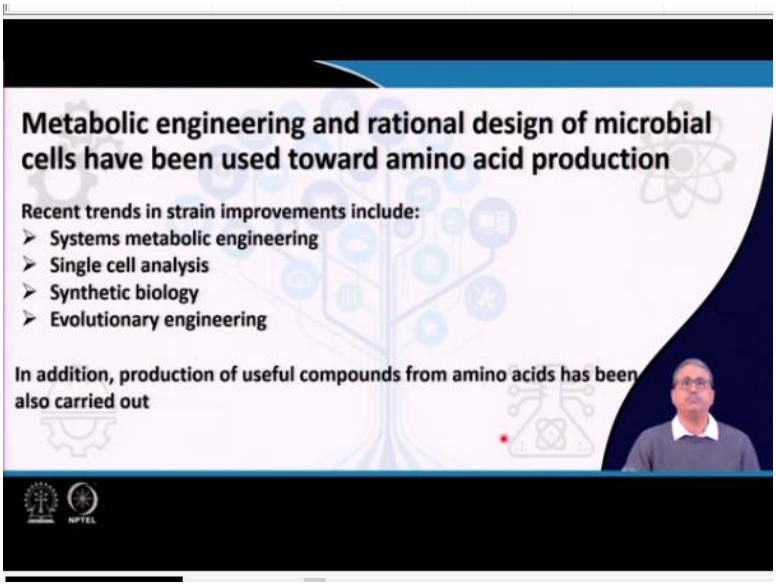


Now metabolic engineering and rational designing of microbial cells have been used towards amino acid production, since the first discovery of the amino acid producing strain *Corynebacterium glutamicum*. Recent trends in strain improvements include systems metabolic engineering, single cell analysis, synthetic biology and evolutionary engineering. In addition, production of useful compounds for amino acids have been also carried out.

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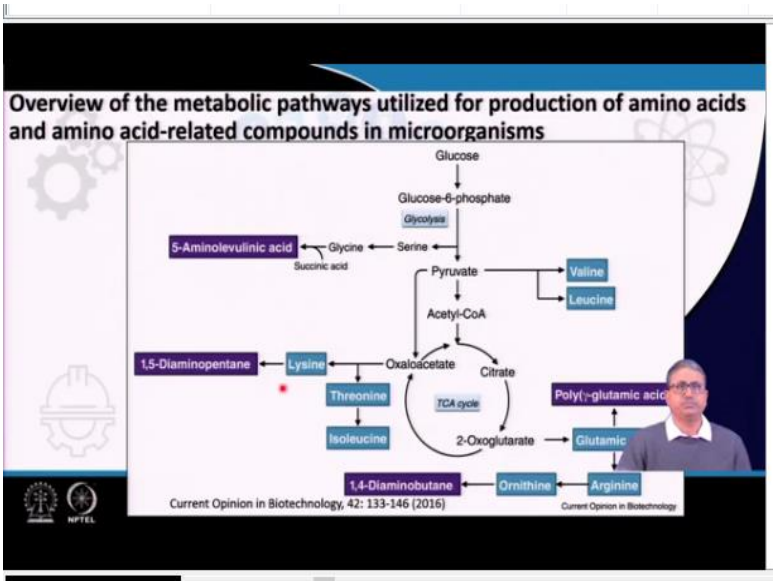


Here is the broad overview of metabolic pathways which are utilized for the production of amino acids and amino acid related compounds through different microorganisms. The blue

shaded are the amino acids which are produced from the central carbon metabolism and the purple shades are basically the amino acid related compounds which are subsequently produced by different modifications of the native pathways.

As can be seen for the amino acids to be produced the central carbon metabolism has a very important and critical role and as we see the glucose is converted to glycolytic reaction through pyruvic acid, acetyl-CoA and then it feeds the TCA cycle through the citric acid, 2-oxogluterate, and oxaloacetate. Now it is to be noted that all these major intermediates which are considered to be the precursors are also in precursors for amino acid synthesis including the pyruvic acid, 2-oxoglutarate, oxaloacetate etcetera.

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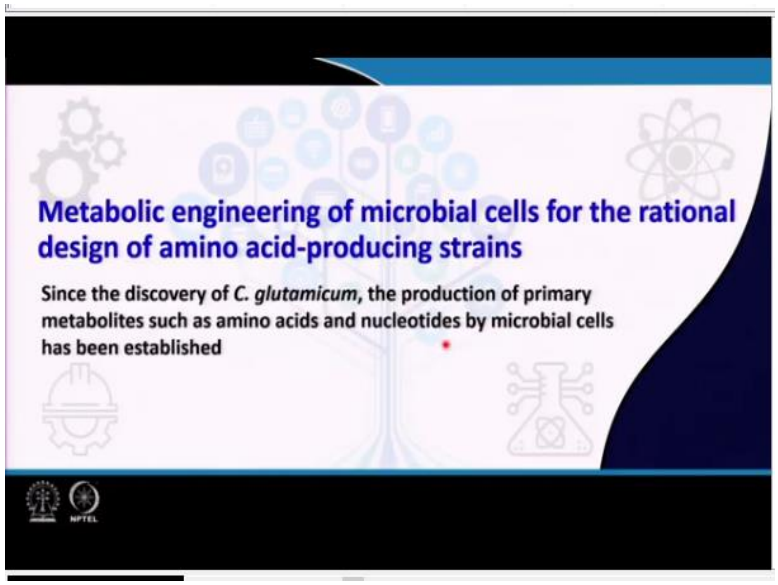


Metabolic engineering of microbial cells for the rational designing of amino acid producing strains have been done in past several decades and since the discovery of the *Corynebacterium glutamicum* the production of primary metabolites such as the amino acids and subsequently different nucleotides were done by microbial cells and these are very well established.

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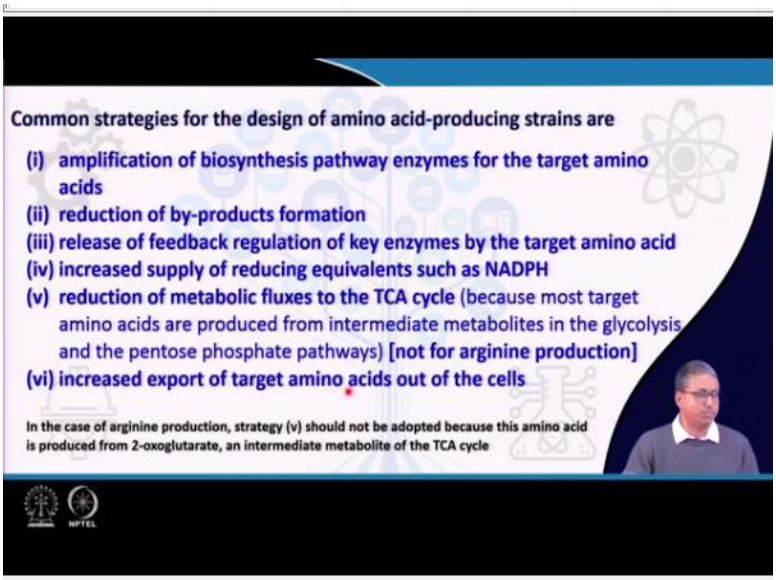


The common strategies for the design of amino acid producing strains are presented here. The major strategies which are generally followed in most of the strain improvement program through metabolic engineering include the amplification of the biosynthetic pathway enzymes for the target amino acids, reduction of by-product formation, release of feedback regulation by key enzymes by the target amino acid.

Increased supply of reducing equivalent such as NADPH, reduction of the metabolic fluxes to the TCA cycle because most target amino acids are produced from intermediate metabolites in the glycolysis reactions and the pentose phosphate pathway and of course for this particular strategy the amino acids which are produced from TCA cycle intermediates are to be exempted and finally the increased export of target amino acids out of the cell.

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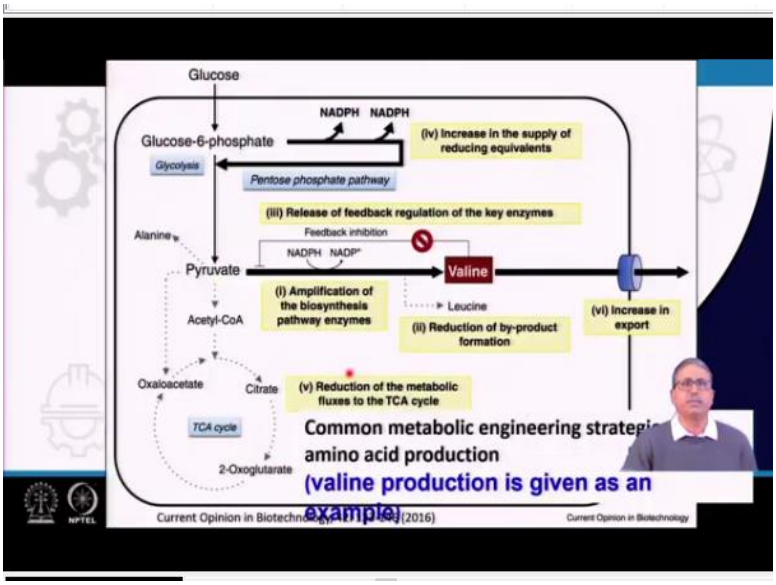

Now if we look at the broad over lay of the different metabolic engineering strategies adopted for improved amino acid production considering the central carbon metabolism, this is the glucose to glucose-6-phosphate, pyruvate, pyruvate is converted to acetyl-CoA and acetyl-CoA feeds into the TCA cycle. We may identify that as already discussed that a number of intermediates of this central carbon metabolism including the pyruvic acid, oxaloacetate or alpha ketoglutarate.

They represent the major starting molecule for the amino acid biosynthesis. So, as a strategy number 1 amplification for the biosynthesis of pathway enzymes considering the valine production as an example, we can see that the pyruvate is used at the starting molecule to produce the valine. So, amplification of the pathway is targeted to achieve higher valine production and as leucine is also produced along during the valine production as a by-product.

So, during valine hyper production or over production of valine often the leucine producing pathway is inhibited or considered that otherwise this may lead to the flux redirection towards leucine which may not be desired then the feedback inhibition control because often these amino acid production pathways are catalyzed by enzymes which are strongly regulated by or strongly regulated through feedback inhibitions to higher concentration of the product inhibits the enzyme which catalyzes the formation of the product.

So, releasing the feedback regulation of the key enzyme is found to be a very important step which is the strategy number 3, then the strategy number 4 is the increasing the supply of the reducing equivalent because most of this amino acid production or almost all amino acid productions require a supply of NADPH. Now arranging that NADPH or a steady supply of the NADPH is essential.

So, in order to produce or supply more NADPH appropriate metabolic engineering strategies can be considered. For example the pentose phosphate pathway or pentose phosphate pathway related enzymes can be overexpressed or can be modified appropriately, so that higher concentration of NADPH is made available inside the cell. Reduction of the metabolic flux to the TCA cycle if the desired amino acid is not produced from the TCA cycle.

For example in case of the glutamate production we see that it is the oxoglutarate or 2-oxoglutarate which acts as the starting molecule for the production of glutamate. So, in that otherwise if that is not the case for other amino acids where TCA cycle is not involved in the production of amino acids. So, reduction of the metabolic flux to the TCA cycle is considered to be one of the important strategies.

And finally in order to export the intracellular amino acid to the culture medium increasing the export system that is the transporters or the membrane transporters are also emphasized.

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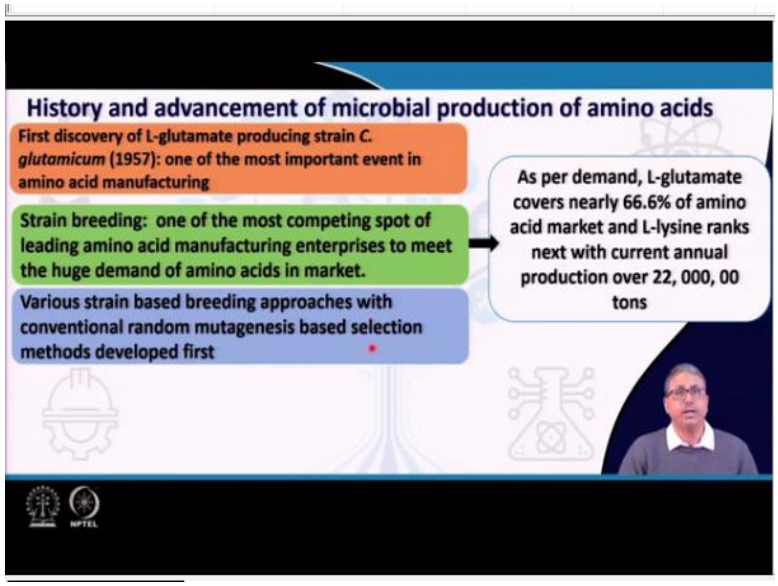


History and advancement of microbial production of amino acids. The first discovery of L-glutamate producing strain *Corynebacterium glutamicum* in 1957 is considered to be one of the most important events in amino acid manufacturing. Strain breeding was found to be the next most important aspects for producing higher concentration of amino acids and many amino acid manufacturing enterprises were interested to meet the huge demand of amino acids in the market.

And as you can see the market for the amino acids were huge like the L-glutamate used to cover around 70% of the amino acid market and the L-lysine ranks the next annual production with annual production over 22 lakh tons. Now various strain based breeding approaches with conventional random mutagenesis based selection methods were developed in the initial days.

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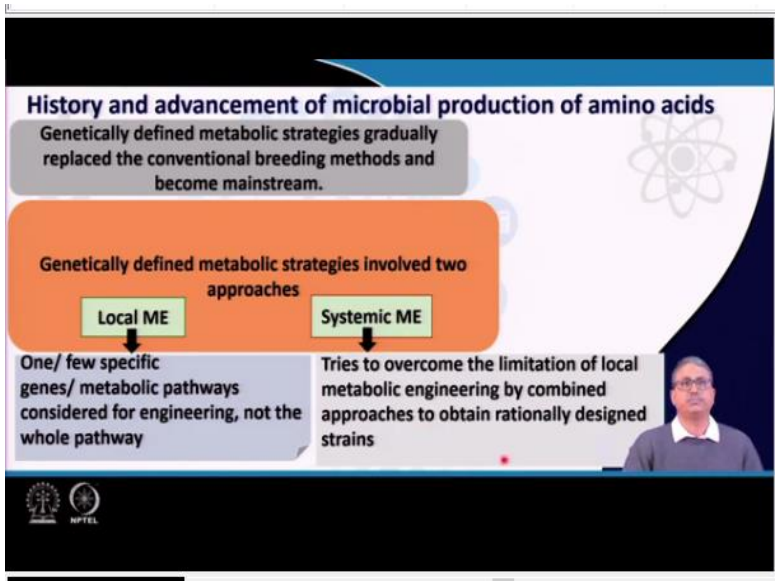


And these were next followed by the improved genetic engineering method with more defined and targeted metabolic engineering strategies. Now genetically defined metabolic engineering strategies involve 2 approaches. One is the local metabolic engineering and another is the systemic metabolic engineering. In local metabolic engineering one or few specific genes or metabolic pathways were considered for the engineering not the whole pathway.

Whereas in case of systemic metabolic engineering it tries to overcome the limitation of the local metabolic engineering by combined approaches to obtain the rationally design strains.

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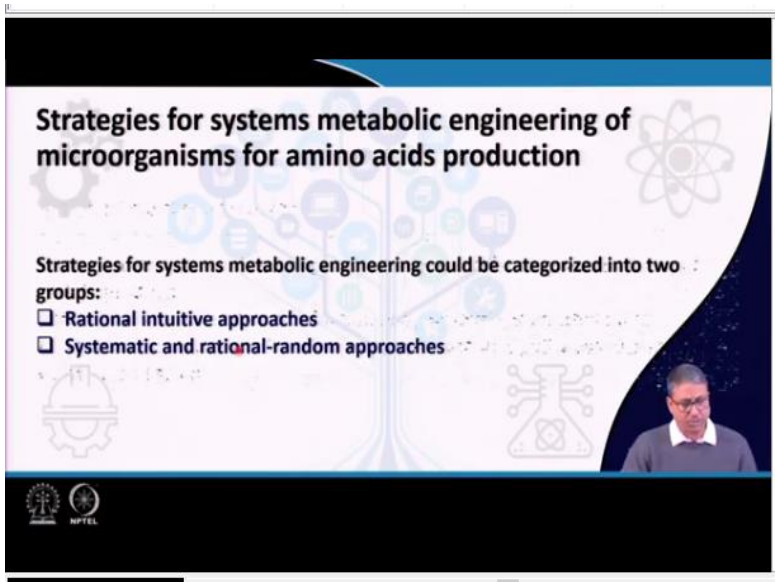


Now the strategies for systems metabolic engineering or microorganisms for amino acid production. Now the strategies for systems metabolic engineering could be categorized into 2

groups. One is the rational intuitive approaches and another is the systemic and rational random approaches.

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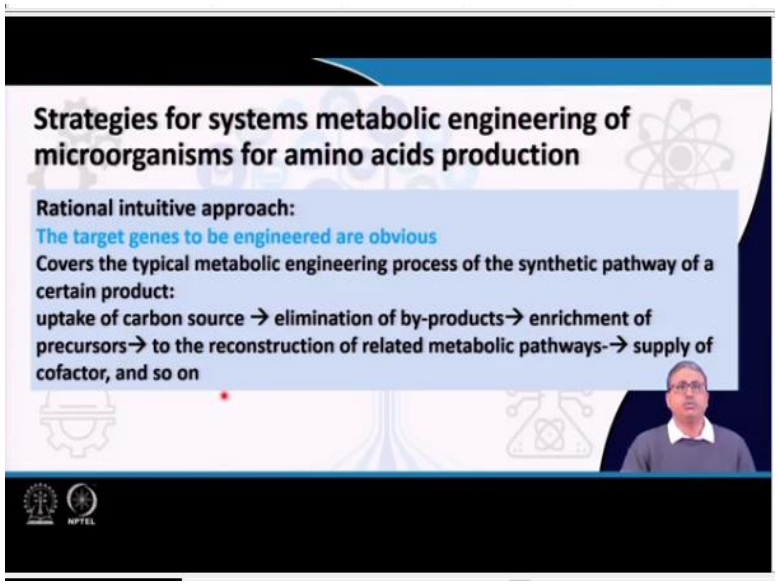


In rational intuitive approach the target genes to be engineered are obvious; they are well identified and it covers the typical metabolic engineering process of the synthetic pathway of certain product including targeting the uptake of the carbon source because in amino acid production the carbon is the basic requirement for the amino acid backbone. Elimination of the byproducts, a enrichment of the precursors to be reconstructed of related metabolic pathways and supply of cofactor and so on.

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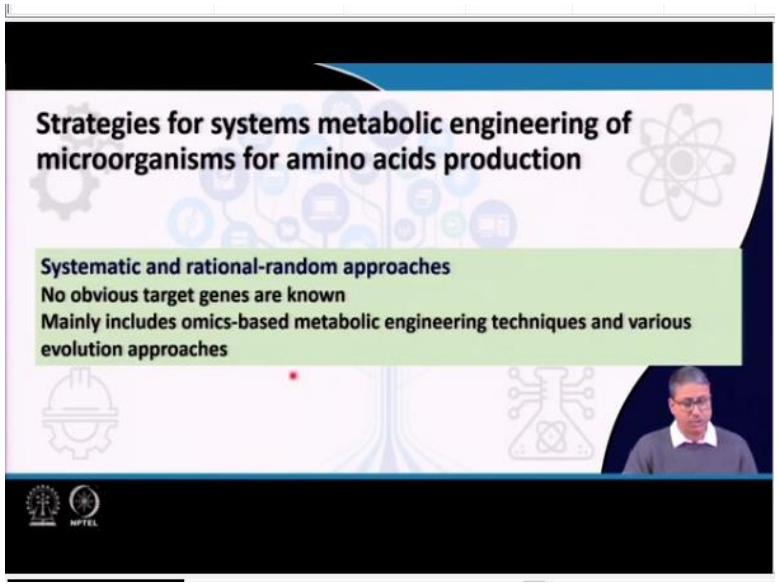


In the systematic and rational-random approaches no obvious target genes are known. So, it is mainly based on omics-based metabolic engineering including the genomics transcriptomics

proteomics and metabolomics to identify the various target molecules and it is not specific to any target gene but it aims to improve the overall cellular metabolism and it includes various evolutionary approaches.

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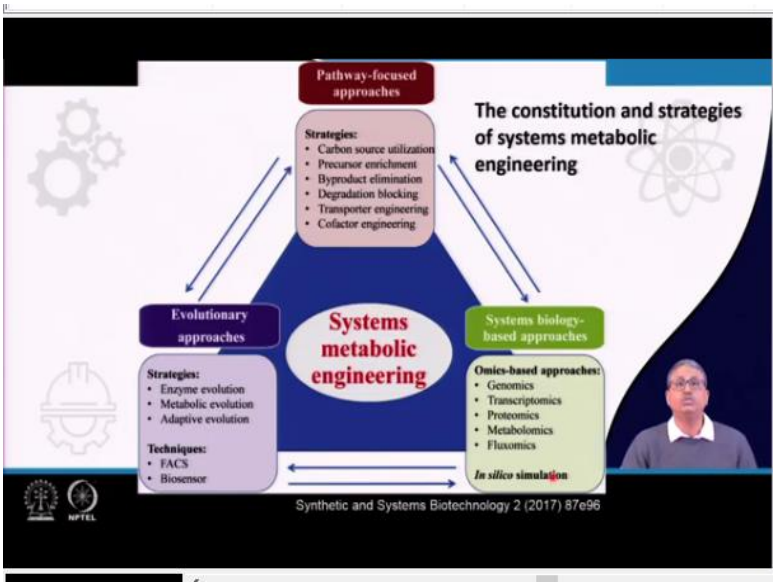


So, here is the broad schematics of the constitution and strategies of systems metabolic engineering which basically includes the evolutionary approach and also the pathway focused approach where the target genes are mostly known and evolutionary approach where the target genes are often not very well defined. But for both these approaches systems biology based techniques and methods including the omics, genomics, transcriptomics proteomics, metabolomics and fluxomix and including the in silico simulations are found to be very important.

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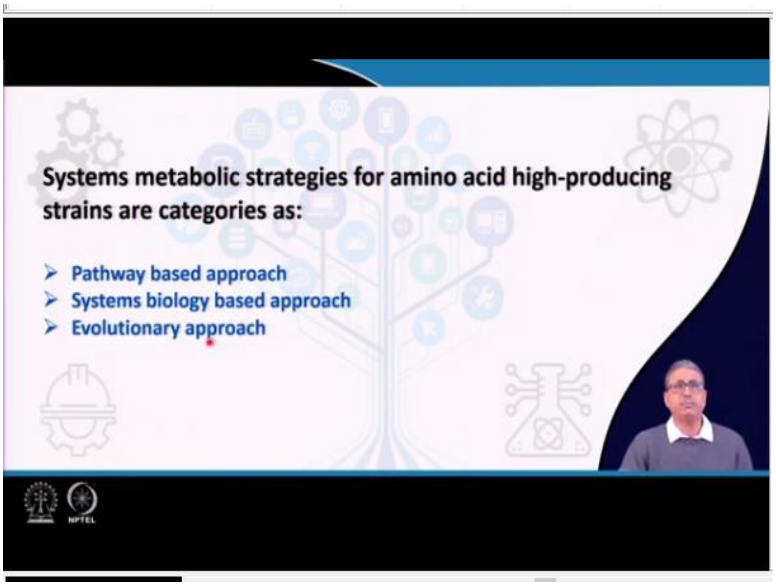

Now systems metabolic engineering for amino acid high producing strains are categorized as pathway based approach, system biology based approach and evolutionary approach.

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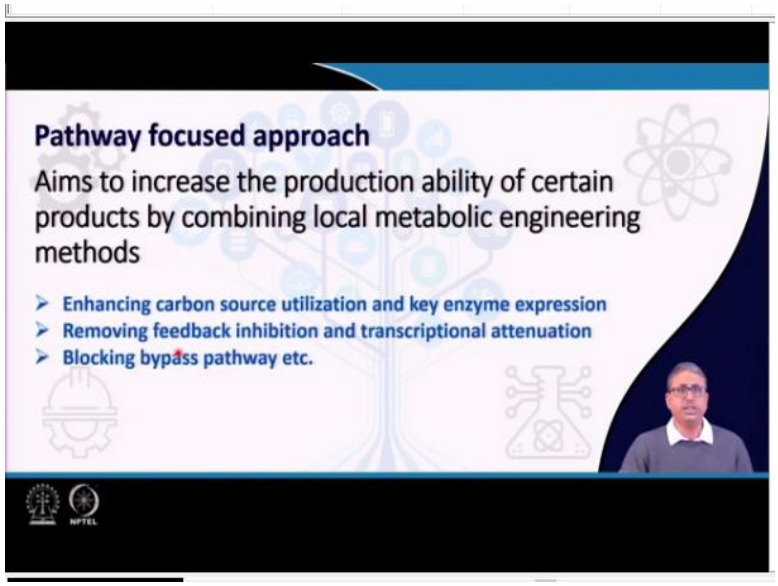


Now in pathway based or pathway focused approach we aim to increase the production ability of certain products by combining the local metabolic engineering methods and this is generally done through enhancing the carbon source utilization and key enzyme expression. Removing the feedback inhibition and transcriptional attenuation and blocking the bypass pathways.

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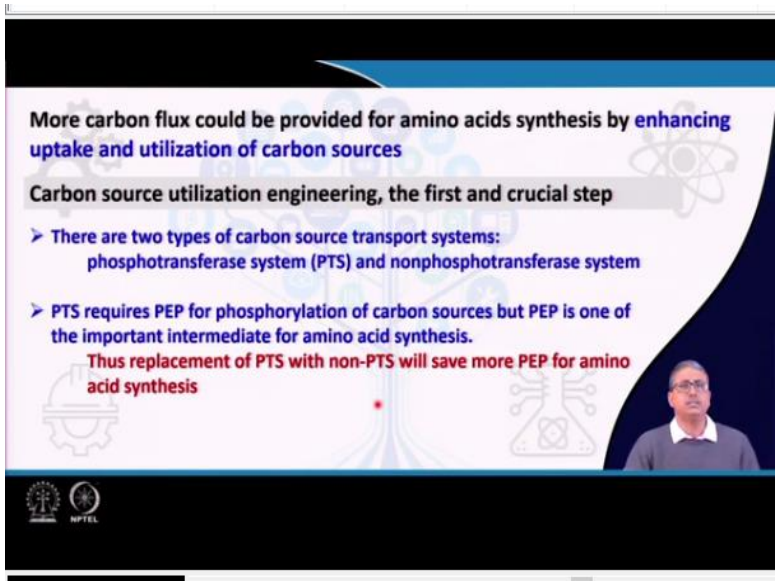


Now more carbon flux could be provided for amino acid synthesis by enhancing uptake and utilization of carbon sources and carbon source utilization engineering the first and crucial step can be achieved through 2 mechanisms. One that there are 2 types of carbon source

transport systems, one is the phosphotransferase system PTS which is dependent on the phosphoenol pyruvate supply and a non phosphotransferase system.

Now the PTS phosphotransferase system requires phosphoenol pyruvate for phosphorylation of the carbon sources, but it is also true that PEP is one of the important intermediate for the amino acid synthesis. Therefore replacement of the PTS that a phosphotransferase system with non PTS transporters for the carbon sources has been found to save some phosphoenol pyruvate for amino acid synthesis.

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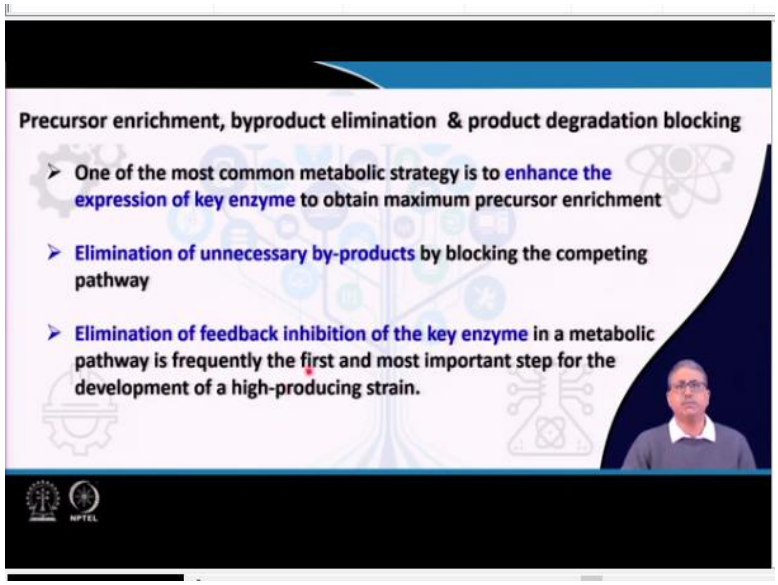


The next is the precursor enrichment followed by byproduct elimination and product degradation blocking. Now one of the most common metabolic strategy is to enhance the expression of key enzymes to obtain the maximum precursors involved in the production of the particular amino acids. Elimination of unnecessary byproducts is also a very important method through blocking the competing pathways.

And finally the elimination of the feedback inhibition of the key enzymes in the metabolic pathway is found to be one of the first and most important steps for the development of high producing strains.

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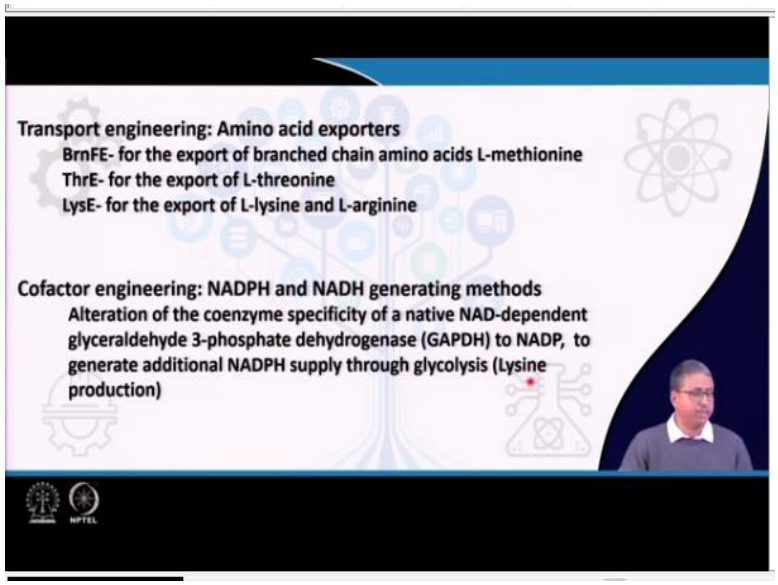


Along with these strategies transport engineering of amino acids that means the transporters which are involved in exporting the amino acids outside the cell are also targeted including the BrnFE transporter for the export of branch and amino acids like L-methionine etcetera also. ThrE for the export of L-threonine and LysE for the export of lysine and arginine. Cofactor engineering is also found to be another important method.

That includes the NADPH and NADH generating methods. Alternation of the coenzyme specificity of the native NAD dependent glyceraldehyde 3-phosphate dehydrogenase to NADP to generate additional NADPH supply through glycolysis has been found to be very effective in lysine production.

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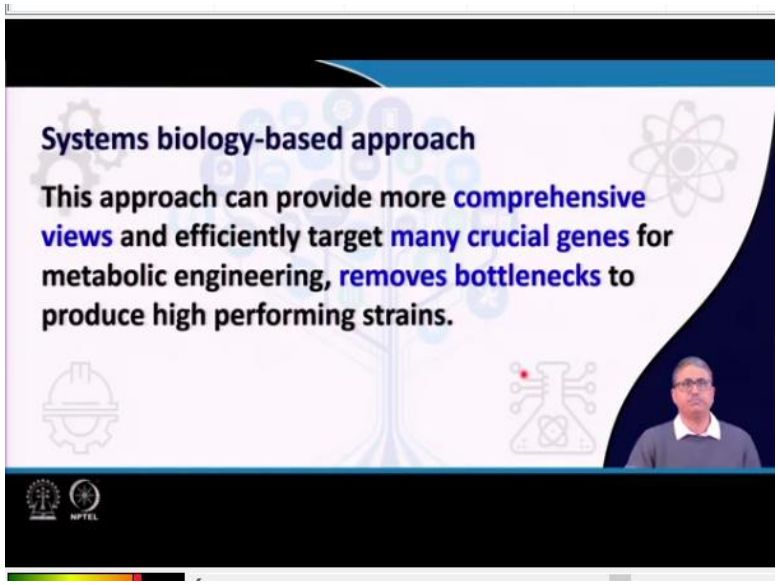

The next is the systems biology based approach. In systems biology based approach we see that it can provide more comprehensive views and efficiently target many crucial genes for metabolic engineering, it also removes the bottlenecks to produce high performing strengths.

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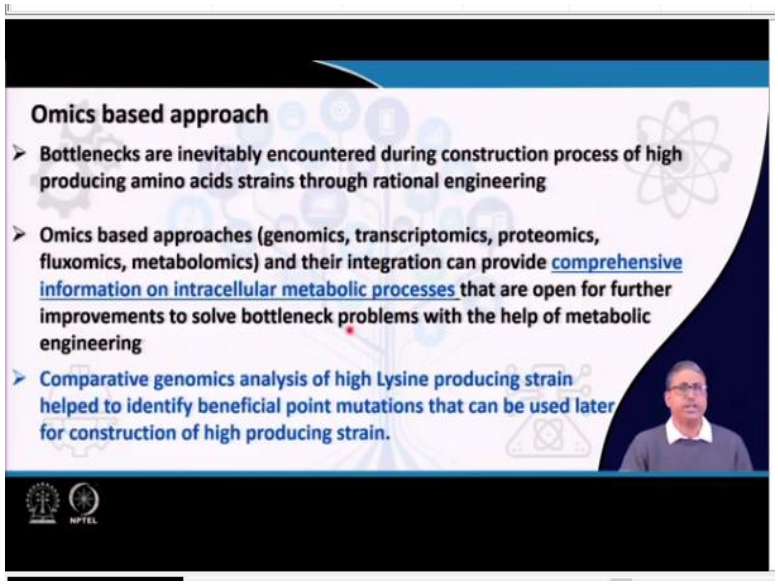


A number of omics-based approaches have been used in the past decades and during the course of the rational designing the bottlenecks of amino acid productions are inevitably encountered through the construction process of high producing amino acid strains and different omics-based approaches including the genomics, transcriptomics etcetera and their integration has been found to be very effective to provide the comprehensive information on intracellular metabolite processes or metabolic processes that are open for further improvements to solve the bottleneck problem with the help of metabolic engineering.

Comparative genomics has also been found to be very effective particularly with respect to high lysine producing strain and it help to identify the beneficial point mutations that can be used later for construction of high producing strains.

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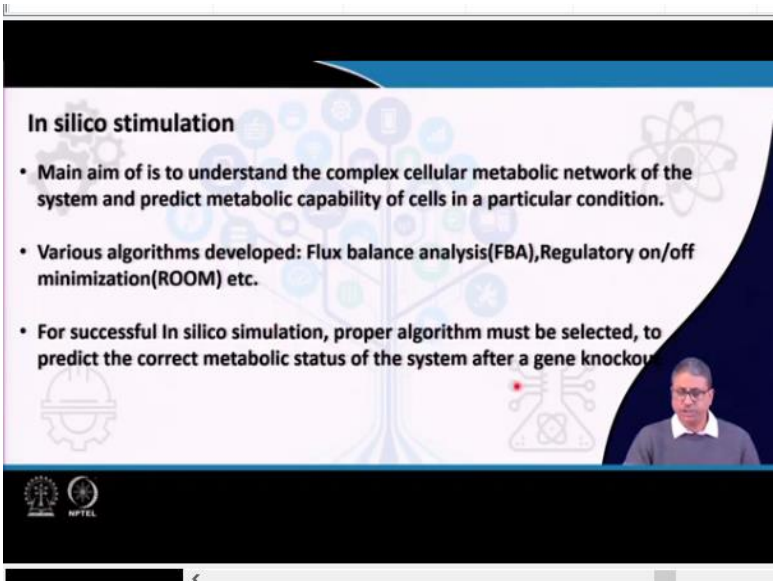


In silico stimulation, the main aim of in silicon stimulation is to understand the complex cellular metabolic network of the system and to predict the metabolic capability of cells in a particular condition. Various algorithms are developed, flux balance analysis FBA, regulatory on, off minimization or ROOM etcetera have been developed. For successful in silico simulation proper algorithm must be selected to predict the correct metabolic status of the system after a gene knockout.

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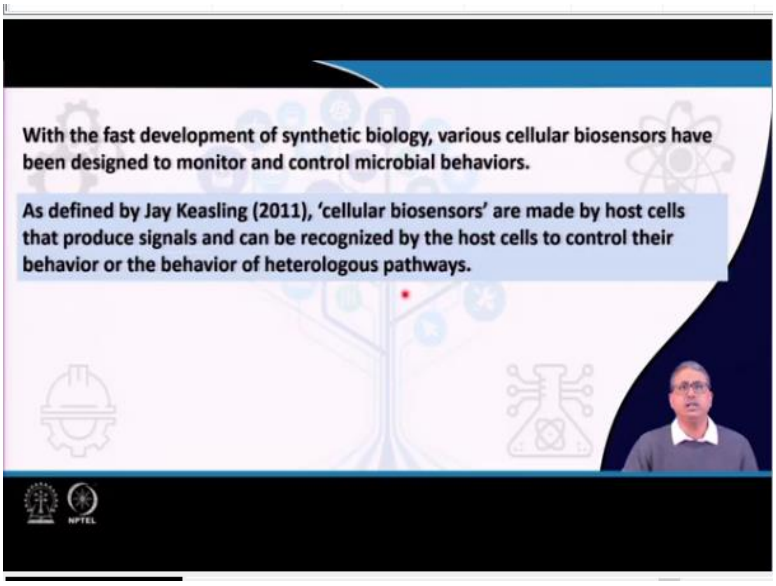


With the first development of synthetic biology various cellular biosensor have been designed to monitor and control microbial behaviour s and as defined by Jay Keasling the cellular biosensors are made by host cells that produce signals and can be recognized by the host cell to control their behaviour or the behaviour of the heterologous pathway.

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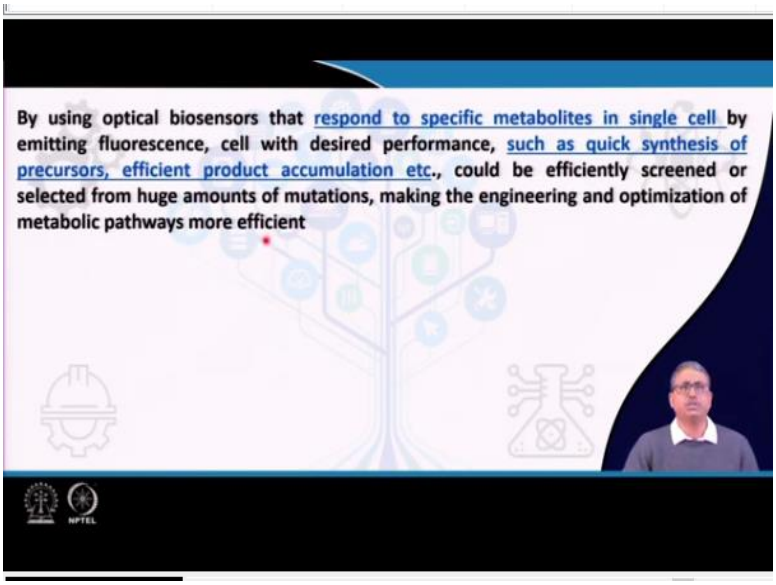


Now by using optical biosensors that respond to the specific metabolites in a single cell by emitting fluorescence, cells with desired performance such as the quick synthesis of the precursors or efficient product accumulation etcetera could be efficiently screened or selected from the huge number of mutations, making the engineering and optimization process for the metabolic pathway improvement more efficient.

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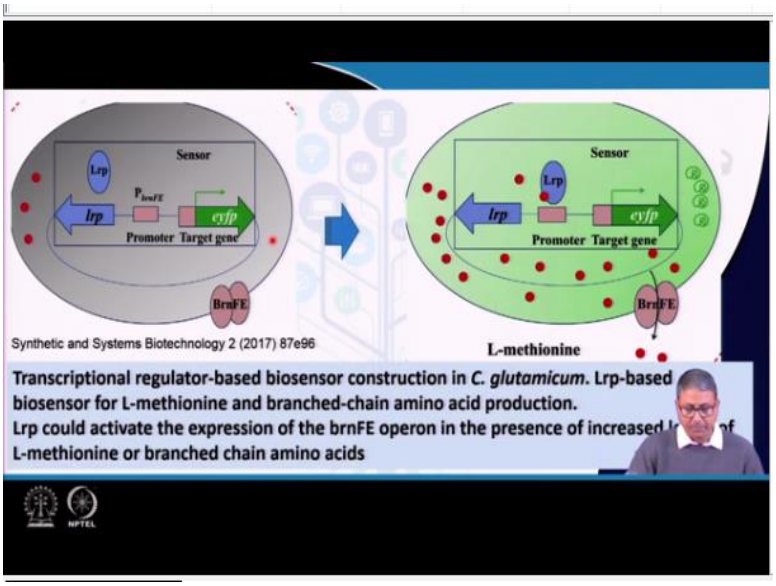


As we can see here that for the efficient production of methionine or other amino acid it has been found that the transcriptional regulator based biosensor construction has been very useful. Now this Lrp based biosensor for L-methionine and other branched chain amino acids are found to be activated and once they are activated they can bound to the brnFE promoter in the presence of the increased level of the methionine and effectively producing more fluorescence signals.

So, it means any single cell or a mutant who is producing more amount of methionine than the other cells can be readily detected and through fluorescent activated cell sorter or similar techniques. Those efficient cells which are able to produce more amount of particular amino acid compared to other cells can be readily identified and selected and segregated. So, because as more amount of amino acid like methionine is produced the Lrp is able to bind that.

And then the Lrp bound with the amino acid methionine can bound to the promoter site and express the relevant the fluorescent producing genes and then these cells can be identified readily.

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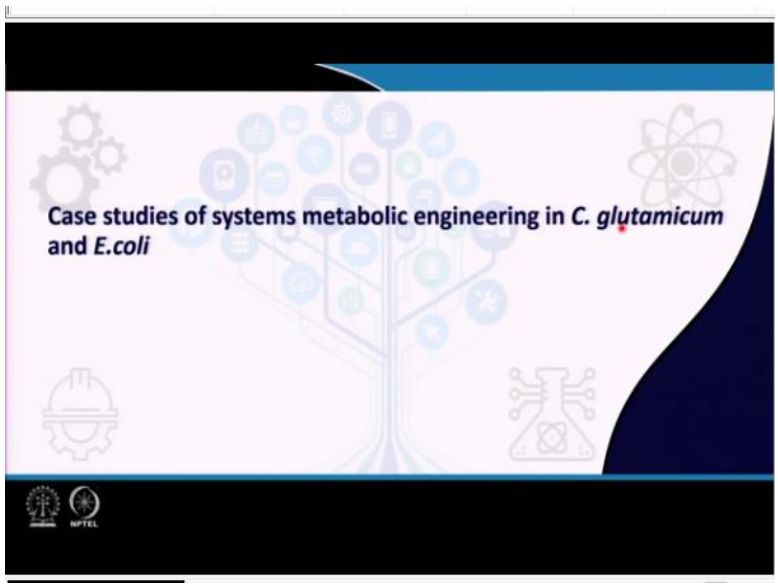


Now we will discuss about a particular metabolic engineering strategy which is considered to be one of the best case studies for system metabolic engineering in *Corynebacterium glutamicum.*

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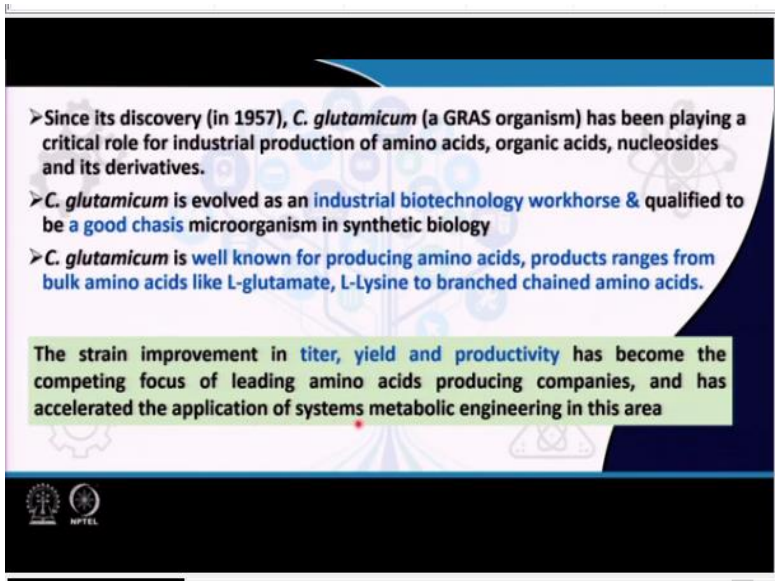


And since its discovery in 1957 *Corynebacterium glutamicum* which is considered to be a GRAS category organism that is generally regarded assay has been playing a critical role for industrial production of amino acids. Organic acids, nucleotide and its derivatives. Now *Corynebacterium glutamicum* is evolved as an industrial biotechnology workhorse and qualified to be a good chasis microorganism in synthetic biology.

This organism is well known for the production of amino acids products ranges from bulk amino acids like L-glutamate, lysine to branched chain amino acids. The strain improvement in titer, yield and productivity for this *Corynebacterium glutamicum* has become the competing focus of leading amino acid producing companies and has accelerated the application of systems metabolic engineering in this area.

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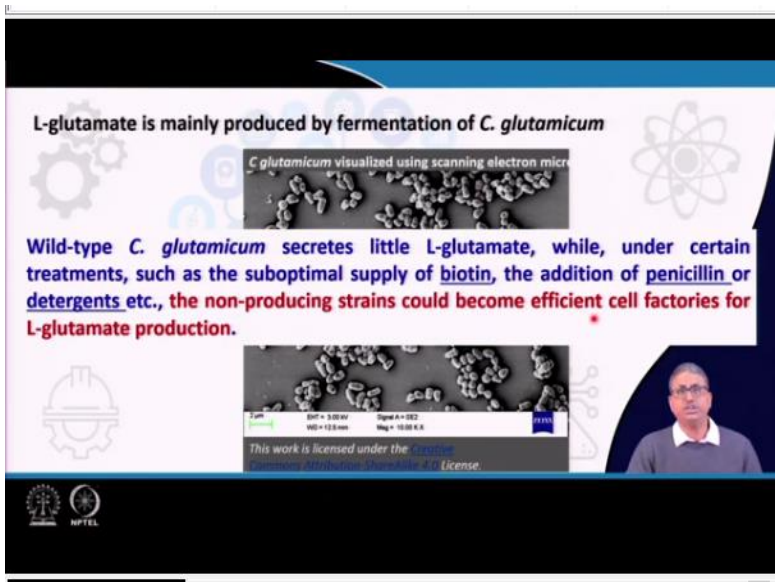

Now L-glutamate is mainly produced by fermentation of *Corynebacterium glutamicum*. Now this Corynebacterium glutamicum one type of strain secretes little concentration of L-glutamate, while under certain treatments such as the sub optimal supply of biotin, addition of penicillin or certain detergents, the non producing strains could become efficient cell factories for the L-glutamate production.

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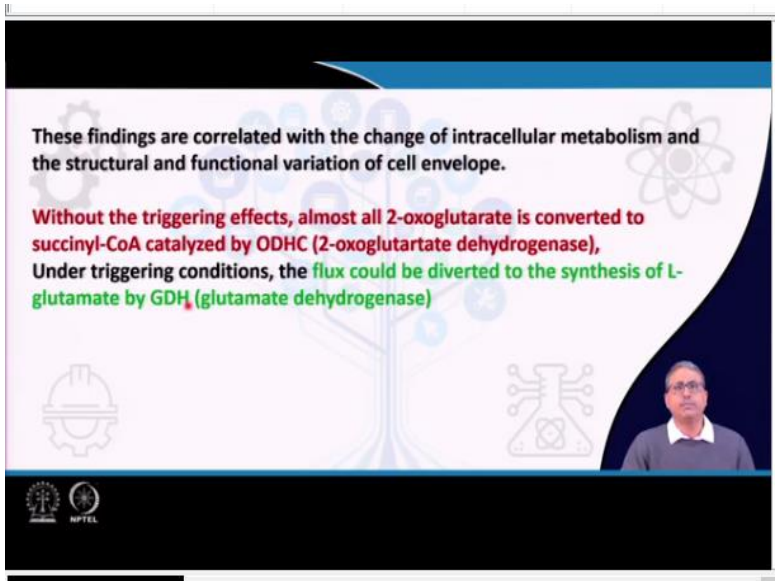


The past research targeted to identify the mechanism of these triggering of higher concentration of amino acid production by different conditions like the detergent amino acid like the penicillin or the biotin and these findings are correlated with the change of intracellular metabolism and the structure and functional variation of the cell envelop. Now it is been found that without the triggering effect, almost all 2-oxoglutarate which is a very important intermediate of the TCA cycle is converted to succinyl-CoA catalyzed by the 2-oxoglutarate dehydrogenase.

However, under the triggering condition the flux could be diverted to the synthesis of L-glutamate by glutamate dehydrogenase.

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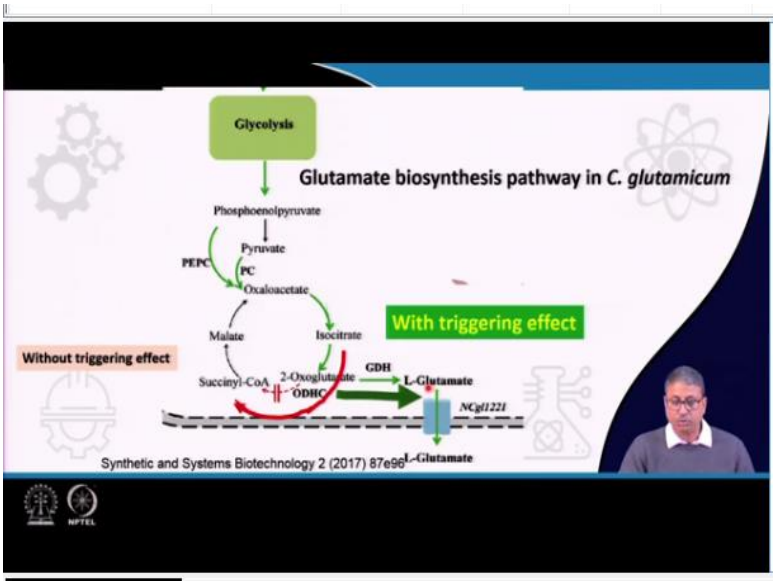


Now if we look at this biosynthesis of glutamate scheme which starts from the glycolysis because the carbon flux is entering through this and then for through the phosphoenol pyruvate and pyruvate it enters to the oxaloacetate and 2-oxaloacetate to oxoglutarate and from oxoglutarate to glutamate it can be channelized. Now without the triggering effect the normal TCA cycle continues and this 2-oxoglutarate can be converted or will be converted to succinyl-CoA.

Now in presence of the triggering molecules like the biotin and penicillin and other detergents this glutamate dehydrogenase enzyme will be highly activated and oxoglutarate will be converted to glutamate very effectively.

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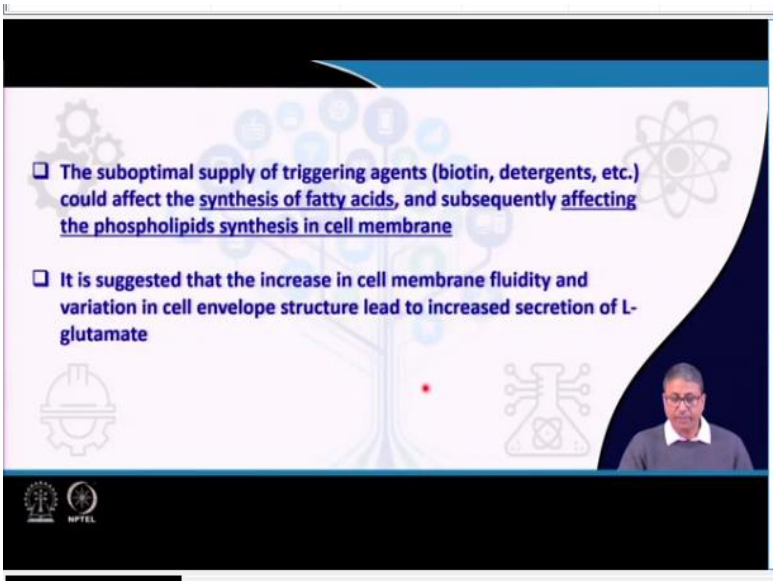

Now the suboptimal supply of the triggering agents like biotin, penicillin and other detergents etcetera could affect the synthesis of fatty acids and subsequently affecting the phospholipid synthesis in cell membrane and it is also suggested that the increase in cell membrane fluidity and variation in the cell envelope structure lead to increased secretion of L-glutamate.

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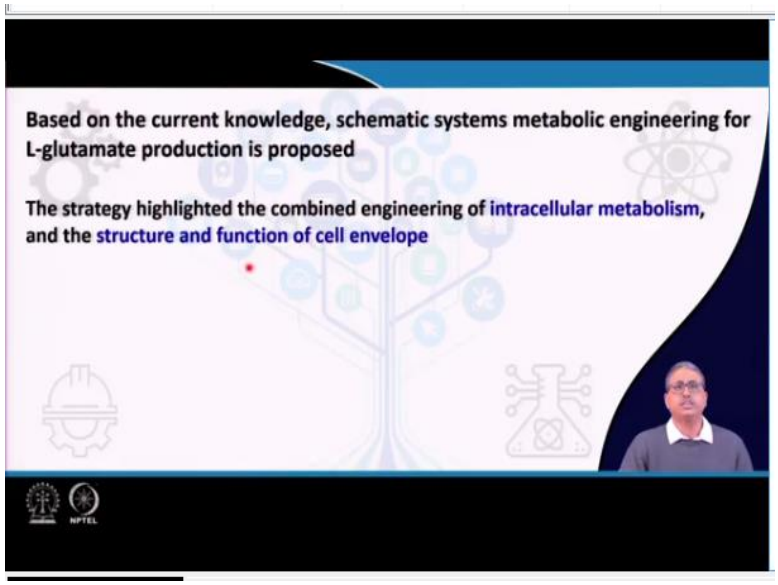


Now based on the current knowledge schematic system metabolic engineering for L-glutamate production is proposed. Now this strategy highlighted the combined engineering of intracellular metabolism and the structure and function of the cell envelope.

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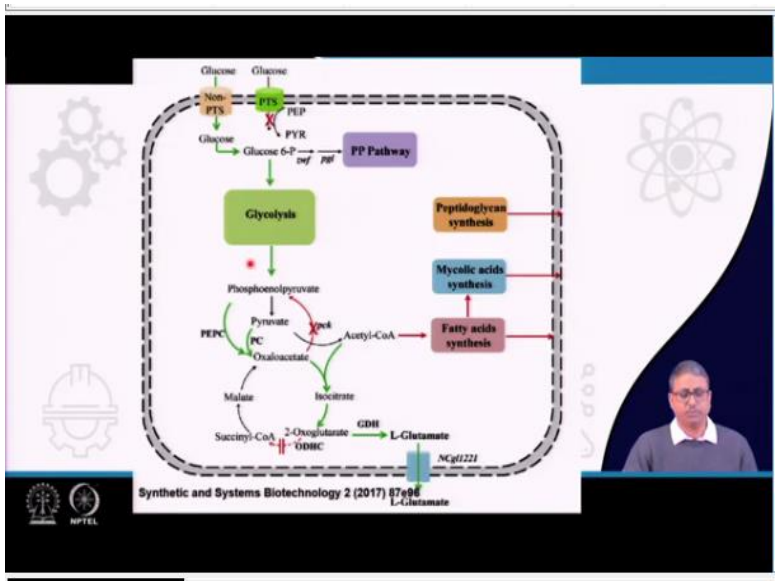


Now as we look into this particular scheme of glutamate production.

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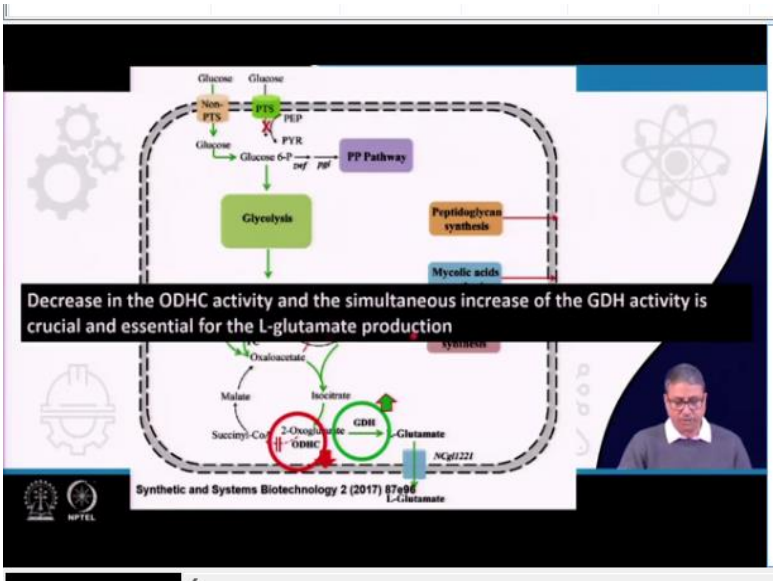


We will be able to see that the decrease in the ODHC activity and this simultaneous increase in the GDH activity is crucial and essential for the glutamate production.

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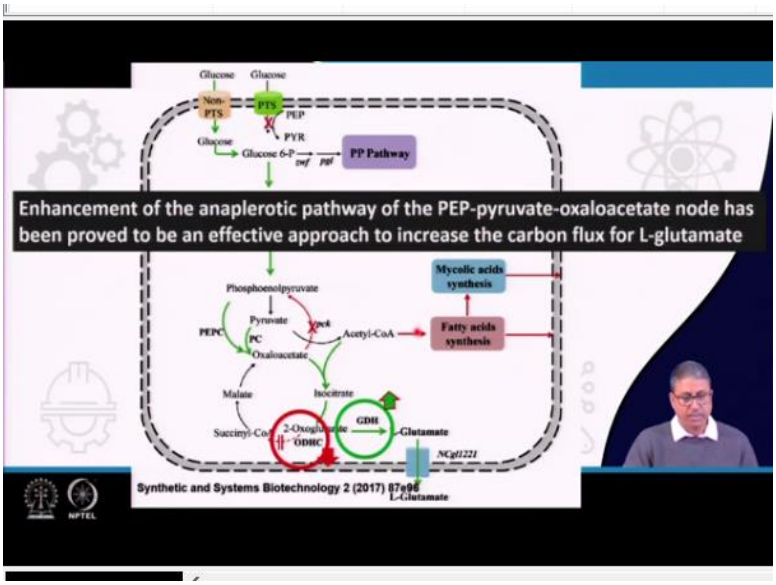


It is also found that enhancement of the anaplerotic pathway of the PEP-pyruvate oxaloacetate node has been found to be an effective approach to increase the carbon flux for the glutamate production.

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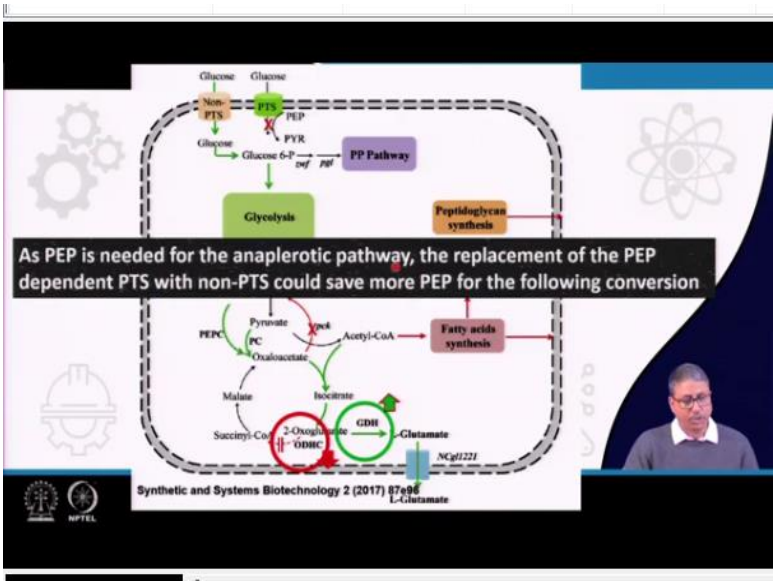


Since PEP is needed for the anaplerotic reaction so here you have the PEP phosphoenolpyruvate and this is required for the anaplerotic reaction to produce more glutamate and the replacement of the PEP dependent PTS. So, as we discussed earlier the glucose can be transported inside the cell through PTS system or non PTS system. The PTS system utilizes the phosphoenolpyruvate.

So, if this PTS dependency is removed by some metabolic engineering strategies that is found to be very effective because relying more on non PTS system for the transport of the glucose makes some more phosphoenolpyruvate available for the anaplerotic reaction.

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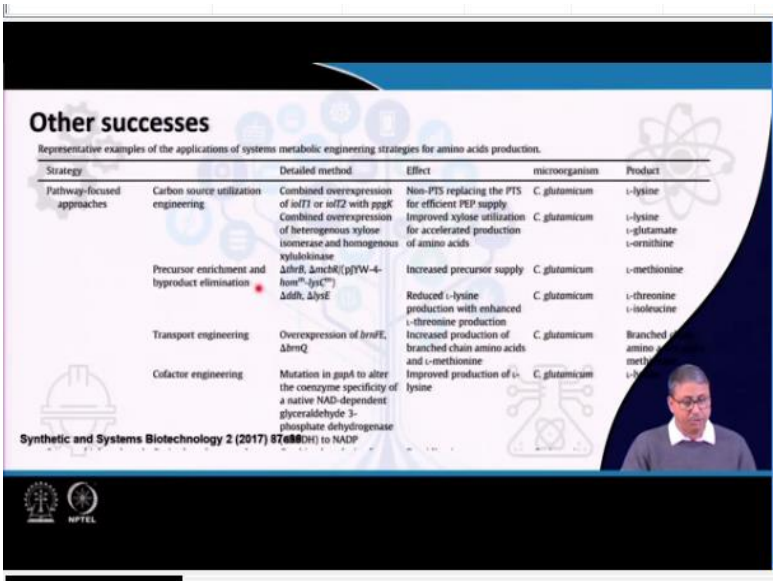


And there are few other successes apart from the glutamate production including the production of lysine using the pathway focused approach.

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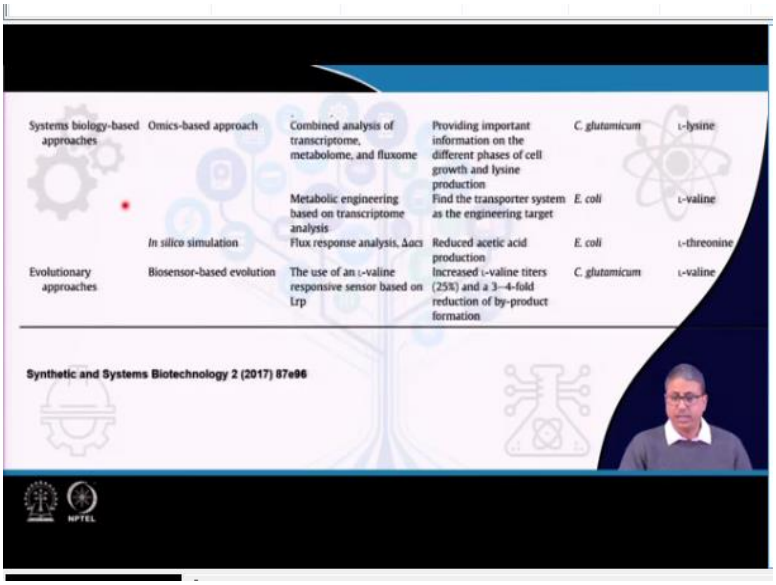


And valine, threonine and different other aromatic amino acids as well through system biology based approach and evolutionary approaches.

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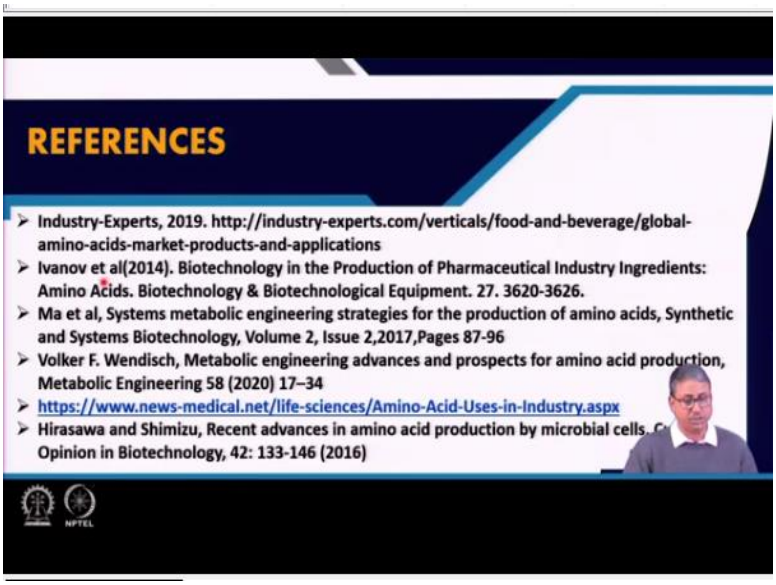


Now for this lecture these are the relevant references particularly the systems metabolic engineering strategies for the production of amino acids and recent advances in amino acid production by microbial cells would be very useful.

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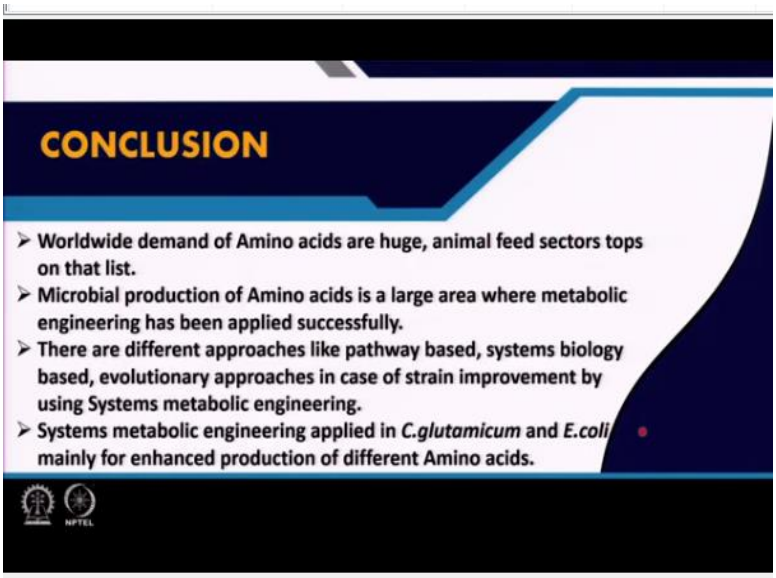


In conclusion worldwide demand for amino acids are huge, animal feed sectors top on that list. Microbial production of amino acids is a large area where metabolic engineering has been applied successfully. There are different approaches like pathway based, system biology based, evolutionary approaches in case of strain improvement by using systems metabolic engineering. Systems metabolic engineering applied in *Corynebacterium glutamicum* and *Escherichia coli* in particular mainly enhanced the production of different amino acids, thank you.