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Review Article

Metabolic Engineering of Bacteria for Food Ingredients

Abdul M[1]*, Areej A[2], Hasnat A[3] and Ghazanfar S[4]

[1]Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Faisalabad, Pakistan

[2]Department of Agriculture, Muhammad Nawaz Sharif University of Agriculture, Multan, Pakistan

[3]Department of Medical Imaging Technology, Riphah International University, Faisalabad, Pakistan

[4]National Institute for Genomics and Advanced Biotechnology, Islamabad, Pakistan

***Corresponding author:** Manan Abdul, Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Faisalabad, Pakistan

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Abstract

Bacteria produces metabolites and their metabolic pathway can be manipulated to produce good food ingredients. These ingredients includes different amino acids, vitamins, carbohydrates, organic acids aroma compounds and bacteriocins. Different genes in different strains of bacteria are identified and harnessed to attain food ingredients and results of manipulation were satisfying. The metabolic pathway of lactic acid bacteria is manipulated to produce amino acids such as L-alanine was produced by silencing the gene that encodes alanine racemase. Moreover, low calorie sugars such as mannitol and tagatose were produced. *Lac-lactis* is engineered by silencing *lacC* and *lacD* genes for the production of tagatose.

Keywords: Metabolic engineering; Bacteria; Food ingredients; Amino acids; Carbohydrates; Vitamins; Bacteriocins

Abbreviations

PTA: Phosphotransacetylase; NOX: NADH Oxidase; ALS: α-Acetolactate Synthetase; PDHc: Pyruvate Dehydrogenase; ALDB: α-Acetolactate Decarboxylase; ADH: Alcohol Dehydrogenase; ACK: Acetate Kinase; DR: Diacetyl Reductase

Introduction

Metabolic engineering is modification and alteration for production of important products or improvement of cellular properties by changing the biochemical reaction or adding the genes of interest by the help of recombinant DNA technology [1,2]. Thus, analysis and variation of metabolic pathways are main aspect, which are dealt in metabolic engineering. In analysis technique such as Metabolic Flux Analysis and other experiments are performed. These techniques help in understanding the cellular response upon genetic modification and environmental change. Modification or alteration includes the use of recombinant DNA technology tools. In the production of food, fermentation play a key role and bacteria are key components of fermentation. Thus, metabolic engineering can be used to modify and enhance the activity of bacteria to produce useful products. Products produced by bacteria are used as catalyst in the production of organic acids, vitamins, amino acids, low calorie sugar products and aroma compounds. Food additives, which are produced by bacteria, can be added into the food as a flavours enhancer, emulsifier, thickener, preservative, and other nutritional supplements. A functional food is defined as a diet that enhance body functions beyond nutritional effects such as health improvement and lessening of disease risk [3]. Vitamins, polysaccharide and low-calorie sugars are main functional food ingredients. Metabolic engineering of *E.coli*, *Corynebacterium glutamicum*, *Bacillus subtilis* can be done to improve and modify the new or novel product with an increased efficacy. The criteria of selection of microorganism for metabolic engineering includes GRAS status, gene transfer methods, rapid growth on cheap carbon and nitrogen source, defiance to bacteriophage attack, ability to metabolize a vast range of carbon source , addition of genes and production at industrial level.

Amino Acids

A variety of amino acids is used in the food and they have great potential in the food. Amino acids are used as flavours enhancers, nutritional supplements, sweetener and as therapeutics.

L-lysine

Corynebacterium glutamicum plays an important role in the synthesis of L-lysine and L-glutamate. *C. glutamicum* strains, which are obtained by classic mutagenesis, are often less stable. There is information about the gene for lysine synthesis. *lysC*, *hom* and *pyc* for aspartokinase, homoserine dehydrogenase and pyruvate carboxylase respectively are three allele, which are responsible for the synthesis of L-lysine. With the help of metabolic engineering, these genes were added into *C. glutmicum* strain. The addition of these gene resulted in high level of lysine production. Mutant and gene in pentose phosphate pathway enzyme 6-phosphogluconolate dehydrogenase production was introduced for improving the cofactor NADPH. Pentose phosphate pathway flux was raised by 8% and 15% increased yield of lysine was observed. *C. glutmicum* shows higher yields in the presence of glucose as compared to sucrose. The reason was fructose-1,6-bisphosphatase activity that limited the lysine yield in the case of sucrose. Thus, *fbp* gene, which encodes for an indigenous fructose-1,6-bisphosphatase was overexpressed and yields of lysine were improved in the case of sucrose as a carbon source. This *fbp* decreased the amount of fructose-1,6-bisphospate that cause inhibition of 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase. *C. glutmicum* cannot assimilate lactose, glactose and starch thus genes that are helpful in assimilation of these from *E. coli* were isolated and added into *C. glutmicum*. Introduction of *amyE* gene from *S. gresius* spp. into *C. glutmicum* resulted in higher yields of lysine. Following figure shows metabolic engineering of *C. glutmicum* metabolic pathways [4] (Figure 1).

Solid lines represent single and dashes represent multiple steps. Bold bar on arrow show that enzyme is blocked. Thick arrow shows that, that enzyme is overexpressed. Bold arrows denote amplified conversions and bold, grey, broken up arrow

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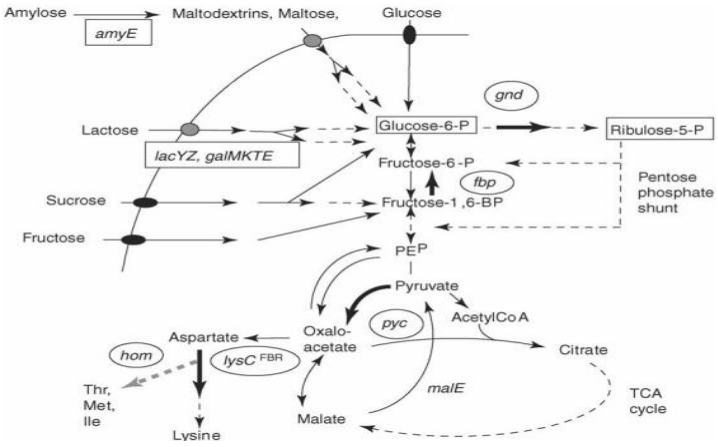

Figure 1: L-lysine.

the decreased conversion by homoserine dehydrogenase. Next to enzymatic reactions, endogenous and heterologous gene names are noted in rings and squares, correspondingly. Movement via phosphoenolpyruvate-dependent transport systems is portrayed in black ovals, transportation via further systems in grey rings.

Amino acids produced from Lactic Acid bacteria

Lactic acid bacteria produce different types of amino acids. L-alanine is produced from a metabolically engineered strain of *Lactococcus lactis*. *B. sphaericus* alanine dehydrogenase enzyme was expressed in a strain, which was deficient of lactate dehydrogenase. L-alanine was produced by silencing the gene that encodes alanine racemase [5].

Organic Acids

Bacteria can produce organic acids by fermentation process. This is very important, as acids are use as acidulants in food industry. Organic acids are also used as flavour enhancer, sweetener, preservatives and beverages ingredients. *E. coli* produce succinate, lactate, ethanol, acetate and formate by the formation of sugar [6]. Anaerobic fermentation performs two main functions, by substrate level phosphorylation, they produce energy in anaerobic environment and by closing redox balance, they provide regeneration source of NAD+. With respect to redox characteristics before mentioned products (succinate, lactate, ethanol, acetate and formate) can be added into three groups on the basis of fermentation pathway. 1) Total formation of redox equivalent by fermentation pathway (e.g. fumarate); 2) Total production of redox equivalent (e.g. pyruvate); 3) Total assimilation of redox equivalent (e.g. succinate). For the production of organic acids by metabolic engineering of bacteria strategies are focused on modification of carbon exchange ratio at 3 points i.e. acetyl coenzyme A, phosphoenolpyruvate and pyruvate.

Pyruvic acid

Escherichia, *Corynebacterium, Enterococcus* and *Pseudomonas* produce pyruvic acid by the fermentation of sugars [7]. But most efficient and satisfying results were observed in the case of *E. coli*. It produced maximum amount of acid in minimum span of time. The strategy was to limit pyruvate dehydrogenase complex activity by providing it limited amount of pyruvate under anaerobic conditions [8]. A strain of *E.coli* named as *E.coli* W1485*lip2* was lipoic acid auxotroph and lipoic acid was found to be cofactor of pyruvate dehydrogenase. This strain produced 25.5g/L of pyruvate from 50g/L glucose in thirty-two hours. Pyruvate production was further improved by mutating F_1-ATPase and the resultant strain was named as TBLA-1 [9]. This TBLA-1 produced 30g/L of pyruvate from 50g/L of glucose only in 24hrs. But due to lower energy production, the growth was decreased to 67%. The increased in the pyruvate production was thought to be due to some glycolytic enzymes [10]. Although glycolytic flux was increased due to decrease in production of ATP, the physiological mechanism causing these changes was not identified. Recently it is observed that glycolytic flux is increased by 70% by increasing the hydrolysis of ATP, which indicated that glycolytic flux was controlled by reactions that hydrolysed ATP [11].

Succinic acid

Wild type of *E.coli* strain produce succinic acid in very minute quantity during fermentation. Genetic manipulation is required to increase the succinic acid production and decrease the production of by product. In initial steps, flux was increased in succinate branch by overexpression of PEP carboxylase. This resulted in 12 to 35% increased succinate production. PEP is substrate for glucose transport by phosphotransferase system PTS in *E.coli* [12]. Thus, directing pyruvate to succinate approach can be used. This can be done by mutating *E.coli* strain by a plasmid pTrc99A-*pyc*, which encodes for Rhizobium pyruvate carboxylase. Succinate yield was increased by 17% by this approach [13]. For overcoming on the issue of by products, genes encoding for those by products were mutated. These genes included *ldhA* for lactate and *pflB* for pyruvate and formate. These mutations resulted the new strain name as *E.coli NZN111*. Its growth was poor under anaerobic conditions. When *Ascaris suum* gene for malic enzyme was transformed into *NZN111,* the succinate yield was increased up to 39% [14,15]. Gene encoding for malate dehydrogenase also resulted in higher succinate productions. Due to unknown mutations in chromosome *NZN111*, strain was able to grow in anaerobic conditions [16]. Thus, name of this strain was changed to *AFP111*. It yielded up to 70% of succinate. It was seen that pyruvate carboxylase expression in *E.coli* can 2.7 folds increase in succinate production [17].

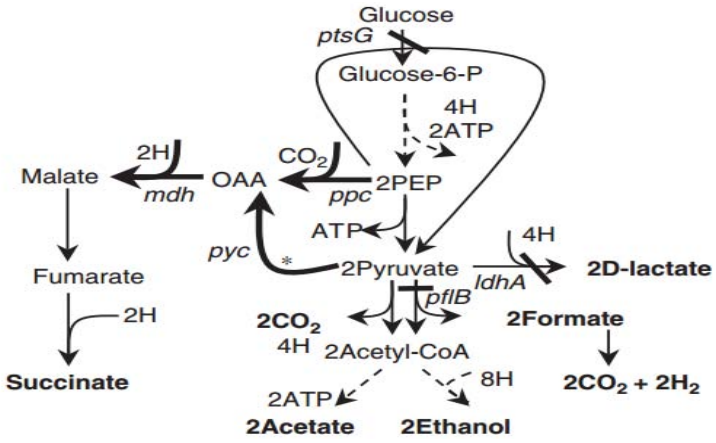

Figure 2: Succinic Acid.

Italics represent the genes; bold letters represent the primary fermentation products and (*) indicate that this step is not present in *E.coli*. Single or several enzymatic reactions are indicated by straight and interrupted lines, respectively.

Acetic acid

Acetic acid is used in vinegar production and as a food preservative. Metabolic engineering of *E.coli W3110* was done to produce acetic acid from glucose [18]. The resultant *TC36* strain produced 34g/L of acetate from 60g/L of glucose. The *TC36* strain was produced by compiling deletions that deactivated oxidative phosphorylation (ΔatpFH) interrupted the action of tricarboxylic acid pathway (ΔsucA) and excluded the native fermentation pathway (ΔfrdBC, ΔldhA, ΔadhE). In redox balance, the loss of carbon and oxygen was decreased due to these mutations.

Lactic acid

L. bulgaricus produce D-lactic acid in high quantities [6]. Other species of lactic acid bacteria also produce L-LAC such as *L. amylophilus*, *L. delbreukii* etc. [19]. One of the drawbacks of using LABs is that they show poor growth rate and their ability to consume pentose is negligible [20]. Metabolic engineering of *E.coli* is performed to produce L and D lactic acid. *E.coli RR1* strain (mutated for *pta* gene) which cannot produce phosphotranscetylase enzyme of Pta-ackA pathway, converted glucose into D-lac and produce small amount of succinate. Mutation in *ppc* (encoding phosphoenylpyruvate carboxylase) made the RR1 to produce L-Lac, which was similar to L-lac produced by LABs. A *pta ldha* mutated strain, which was transformed with L-lactate dehydrogenase encoding gene, produced L-lactate by metabolizing glucose and amount of L-LAC was about 45g/L [21].

Vitamin

Vitamins are said to be nutraceuticals because they act as a cofactor of many enzyme in metabolic processes. They are essential part of human diet.

Vitamin B2 (Riboflavin)

L. lactis and *B. subtilis* have been engineered to produce riboflavin. Rib gene was overexpressed in *B. subtilis* for the production of riboflavin [22]. This was done by replacing promoter from a constitutive phage [23]. A construct of four genes was added into two different sites of genome for obtaining maximum riboflavin. *ribA* gene encodes for a protein, which has GTP cyclohydrate, II activity at C terminal and at N terminal; it has dihydroxy butanone phosphate synthetase activity. *L. lactis* was also engineered with overexpressing *ribA* gene, for the production of Vitamin B2 that encodes for GTP cyclohydrase activity. The production of riboflavin was increased up to 3 folds by overexpression of *ribA* gene [24].

Vitamin C (L-Ascorbic Acid)

2-keto-L-gluconic acid 2-KLG is the product during the synthesis of ascorbic acid. This 2-KLG is transformed into ascorbic acid by esterification and lactonization. Studies are carried out to understand the production of ascorbic acid from different sugars. *Gluconobacter oxydens* is metabolically engineered to produce 2-KLG from sorbitol [25]. Sorbitol dehydrogenase and sorbose dehydrogenase were responsible for the conversion. Thus, genes of these enzymes were transformed into *Gluconobacter oxydens G624*. Another specie of bacteria *Erwinia herbicola* was transformed with a gene for 2-KLG reductase. This reductase converts 2,5-diketo-d-gluconic acid to 2-KLG. *E. herbicola* was able to produce 2-KLG from glucose in a single step. 120g/L concentrations have been achieved from this strain [26].

Vitamin B11 (Folate)

Genes involved in synthesis of folate from guanosine triphosphate were expressed in *L. lactis*. By the help of this approach, folate production was increased three to six folds [25].

Carbohydrates

Polysaccharides, which are indigestible, have a great impact on the growth of microflora in intestine. These are known as prebiotics. Lactic acid bacteria are good source of exopolysaccharides. These polysaccharides enhance texture, mouth feel, taste and stability of food product. EPS exopolysaccharides can be produced by transforming the same genes cluster due to their polymerization and limited supply of precursors [27]. *epsD* gene that encodes for glucosyltransferase is overexpressed in *lactobacillus lactis* to produce EPS. *Fbp* gene that encodes for fructose bisphosphatase enzyme has been overexpressed

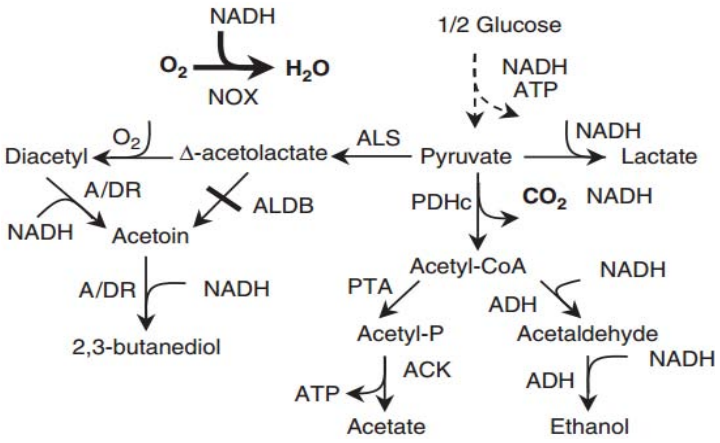

Figure 3: Aroma Compounds.
Abbreviations: PTA: Phosphotransacetylase; NOX: NADH Oxidase; ALS: α-Acetolactate Synthetase; PDHc: Pyruvate Dehydrogenase; ALDB: α-Acetolactate Decarboxylase; ADH: Alcohol Dehydrogenase; ACK: Acetate Kinase; DR: Diacetyl Reductase.

in *Lac.lactis* to increase the production of EPS. Also overexpression of *galU* and *galE* genes in *Lac.lactis* caused an increase UDP-glucose and glactose which are precursors of EPS [28].

Bacteriocins

Bacteriocins enhance the quality of food. Some of lactic acid bacteria produce bacteriocin as secondary metabolites. LABs functions as preservative in food they enhance the shelf life of fermented food. Bacteriocins are proteins, which are synthesized ribosomally and undergoes post-translational modifications [29]. Live microbes can be added into food and they can produce bacteriocins inside the food and preserve it. As these are cheap preservative and most strains are GRAS thus it is very economic to use them in food. Lactococcus strain was engineered to produce pediocin PA-1. Lactococcus also produce lactococinA. The heterologous system of expressing these bacteriocins was used to produce different bacteriocins [30,31].

Low Calorie Sugars

Mannitol, tagatose and sorbitol are regarded as low calorie sugars. They have positive effect on human health because they help in weight loss. Due to this aspect, their demand is very high in market. Tagatose is a prebiotic and low-calorie sweetener. *Lac-lactis* is engineered by silencing *lacC* and *lacD* genes for the production of tagatose. By this genetic modification tagatose-1,6-disphosphate and tagatose-1-6-phosphate were being produced [24]. Dephosphorylation of tagatose-1,6-diphosphate can yield tagatose thus current strategies are focused on this dephosphorylation.

Aroma Compounds

In diary industry, diacetyl is used as an important flavour. *Lac. lactis* has been engineered by deactivating *aldB* gene that encodes for α-acetolactate decarboxylase and activity of NADH oxidase was overproduced for the production of diacetyl [5]. By this strategy, 80% of carbon source was converted into diacetyl. Enzymatic degradation of amino acid yields aroma compound. These amino acids include phenylacetate, indole etc. *Lac.lactis* was engineered by overexpressing GDH gene that encodes for glutamate dehydrogenase. This glutamate dehydrogenase converted amino acids into aromatic compounds.

ME of *Lac.lactis* for the production of diacetyl. Solid lines represent single and dashes represent multiple steps. Bold bar on arrow show that enzyme is blocked. Thick arrow shows that, that enzyme is overexpressed.

Conclusion

Bacteria are good source of metabolites. Their metabolic pathways can be engineered to get desired products and among these products, food ingredients are one of most important. *Corynebacterium glutamicum* plays an important role in the synthesis of L-lysine and L-glutamate. Lactic acid bacteria produce different types of amino acids. L-alanine is produced from a metabolically engineered strain of *Lactococcus lactis*. *B. sphaericus* alanine dehydrogenase enzyme was expressed in a strain, which was deficient of lactate dehydrogenase. L-alanine was produced by silencing the gene that encodes alanine racemase. *L. lactis* and *B. subtilis* have been engineered to produce riboflavin. *Rib* gene was overexpressed in *B. subtilis* for the production of riboflavin.

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