

## 42 Metabolic engineering of lactic acid bacteria for the improvement of fermented dairy products

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### Introduction

The lactic acid bacteria *Lactococcus lactis* and *Streptococcus thermophilus* play a crucial role in the manufacture of dairy products such as cheese, buttermilk and yogurt. In this contribution, different metabolic engineering strategies are described aimed at improvement of the quality of the fermented dairy product. By increased production of diacetyl or acetaldehyde, the particular flavour of respectively buttermilk and yogurt are improved. The health benefits of dairy products are improved by complete conversion of lactose and galactose to, for instance, the sweetener alanine or by induced high-level vitamin-production. The structural properties of fermented products is increased by the production of exopolysaccharides with desired properties.

*L. lactis* is used as starter bacterium in the manufacture of many different cheese types, such as Gouda and Cheddar cheese, and for butter and buttermilk production. It can grow rapidly on milk, converting lactose homofermentively to lactic acid. As nitrogen source it hydrolyzes the milk protein casein by way of an extracellular proteinase. This process of proteolysis continues in ready-made cheese during the ripening process resulting in amino acid-release and subsequent flavour-formation. In addition, some *L. lactis* strains are able to convert the citric acid in milk, resulting in gas (CO<sub>2</sub>) formation and production of the butter flavour, diacetyl [1].

Some *L. lactis* strains produce relevant amounts of exopolysaccharides resulting in high viscosity of the end-product. This process occurs in some Scandinavian dairy products, such as viili and longfil.

*Streptococcus thermophilus* is used as starter for the production of yogurt and for Swiss (Emmentaler) and Italian (Parmesan) type cheeses. It can grow extremely rapid in milk at elevated temperatures up to 45 °C. A special characteristic of the metabolism of this bacterium is that it utilizes only the glucose-moiety from lactose, resulting in fermented products containing residual galactose. The specific flavour compound produced by this bacterium during milk fermentation is acetaldehyde. It is, presumably, produced by conversion of the amino acid threonine into glycine (and acetaldehyde). Yogurt bacteria are known for their ability to produce exopolysaccharides, resulting in a desired, slimy, structure of the fermented product.

## Metabolic engineering of lactic acid bacteria

*Lactococcus lactis* is a well-characterised bacterium on the level of physiology and, especially, molecular biology. All necessary genetic tools have been developed for this organism, including food-grade cloning vectors and selection markers. Its genome has recently been sequenced. This opens the way for application of genetically modified *L. lactis* strains in the very near future.

The metabolism of *L. lactis* is relatively simple with energy metabolism centred around rapid conversion of sugar into lactic acid, and biosynthetic pathways kept to a minimum. This makes the organism an attractive model for metabolic engineering activities. Recently a very efficient and tightly-controlled expression system was discovered in *L. lactis*, involving the production of the antimicrobial peptide nisin. The system, named NICE (nisin controlled expression [2]) has since been applied for controlled expression of a number of desired proteins and enzymes.

*S. thermophilus*, with a smaller genome size than *L. lactis*, appears to have an even more limited metabolic capacity than *L. lactis*. However, its metabolism has not been studied in such detail as *L. lactis* and its genetics are far less developed. The NICE expression system from *L. lactis* has been introduced successfully in *S. thermophilus*.

## Flavour formation

### Diacetyl

For many dairy products, the butter aroma diacetyl is an essential flavour component. It is produced during conversion of the citric acid in milk by specific, citrate-utilising, lactic acid bacteria. In this process, citric acid is converted to pyruvate and, since no reducing equivalents are generated, pyruvate is further converted, in a redox neutral reaction to  $\alpha$ -acetolactate, the precursor for diacetyl.

In most lactic acid bacteria this  $\alpha$ -acetolactate is enzymatically decarboxylated to the metabolic end-product acetoin, but special, aromatic, strains do not contain the responsible enzyme,  $\alpha$ -acetolactate decarboxylase, resulting in accumulation of  $\alpha$ -acetolactate and high production of diacetyl in dairy products.

With this background knowledge in hand and a detailed description and model of (pyruvate) metabolism of lactic acid bacteria (see contribution by Hoefnagel *et al.*), an efficient strategy was designed for *L. lactis* to reroute milk sugar metabolism in the direction of diacetyl production. A combination of a hundred-fold overproduction of NADH-oxidase and disruption of  $\alpha$ -acetolactate decarboxylase turned out to be the most successful [3].

## Acetaldehyde

Acetaldehyde is a major component of the typical yoghurt flavour. It is produced by both yoghurt bacteria, *S. thermophilus* and *Lactobacillus bulgaricus*. The exact mechanism of acetaldehyde production is currently not well established. In principal, there are two major possibilities: (i) From pyruvate via the pyruvate-formate lyase or the pyruvate dehydrogenase reaction, and (ii) From threonine via the threonine aldolase reaction resulting in glycine and acetaldehyde production.

The amounts of acetaldehyde produced by the yoghurt bacteria are small, in the range of 5 to 10 ppm. Recent studies in our laboratory show: (i) a five- to ten-fold increase in acetaldehyde production as a result of extra added threonine (10 mM) to the growth medium, (ii) an almost linear relationship between the level of acetaldehyde produced during milk fermentation and the threonine aldolase activity measured in different *S. thermophilus* strains [4]. This clearly shows the importance of this enzyme in flavour formation in yoghurt. Current work is focussing on metabolic engineering on the level of threonine metabolism to control the flavour generation in yoghurt.

## Production of exopolysaccharides

In several fermented dairy products, lactic acid bacteria are used that contribute to the structural characteristic of the product by the production of exopolysaccharides (EPS). This is the case in yoghurt, where both *S. thermophilus* and *L. bulgaricus* are able to produce these sugar polymers, and also in some Scandinavian dairy products such as viili and longfil. In the latter products *L. lactis* is used as starter bacterium. A strain, isolated from these dairy products, showed EPS-production associated with the presence of a 42 kb plasmid. A specific EPS operon was identified on this plasmid containing 14 genes involved in biosynthesis of EPS from the different nucleotide-sugar precursors. By subcloning of the genes and by homology comparison with known DNA-sequences, the exact role of most of the 14 genes in EPS-biosynthesis was established [5]. Currently, research is focussed on

increasing the production of the different nucleotide-sugars involved in biosynthesis of the EPS-repeating unit, namely UDP-glucose, UDP-galactose, and dTDP-rhamnose. Careful flux-analysis through the pathway of nucleotide-sugar conversion and cloning of the genes responsible for these conversions (*pgm*, *galE*, *galU*, *rfbABCD*), will be a strong basis for metabolic engineering aimed at increased EPS production by lactic acid bacteria [6].

Another approach is to concentrate on the molecular size of the EPS-molecule since its intrinsic viscosity is directly related to (the square of) its size. Growth experiments have already shown that, although no changes in absolute production were observed, a mere two-fold increase in molecular weight could lead to industrially significant changes in the structural characteristics of the fermented products [7]. It should be possible, by cloning the genes responsible for elongation of the polysaccharide-molecule, to control size and structural characteristics of EPS-molecules.

## Health aspects

Many fermented dairy products are claimed to be beneficial for the health of the consumers as a result of specific, health-promoting, activities of the lactic acid bacteria. However, the mechanisms of these, so-called, probiotic effects are in most cases not known and many health claims are not very well substantiated.

Two clear examples of health-promoting activities by lactic acid bacteria are described: (i) removal of lactose (and galactose) and production of L-alanine, and (ii) the production of vitamins.

## Lactose/galactose removal

Fermented dairy products such as buttermilk and yoghurt contain high amounts of residual lactose. In addition, yoghurt contains considerable amounts of galactose, due to the inability of the yoghurt bacteria to ferment this sugar. High lactose content is undesirable for all lactose-intolerant human beings, which make up a large part of the world population. Too high galactose consumption, especially in combination with ethanol consumption, can lead to accumulation of toxic galactitol (major cause of cataract) in human tissue cells.

In yoghurt and buttermilk, lactose utilisation proceeds as long as the external pH allows metabolic activity of the lactic acid bacteria. When so much lactic acid is produced that the pH has reached 4.0 in yoghurt and 4.5 in buttermilk, the yoghurt (*L. bulgaricus*) or buttermilk bacteria (*L. lactis*) cease their metabolic activity and lactose is no further utilised. By rerouting metabolism of the lactic acid bacteria towards production of more pH-neutral components, further conversion of lactose will become possible. In this respect, the recent finding that in *L. lactis* sugar metabolism can be completely converted towards L-alanine production

instead of lactic acid [8], by cloning alanine dehydrogenase into this microorganism, opens interesting possibilities to effectively remove lactose. Not only will the fermented product be free of lactose, but sweetness and general taste will be enhanced by the formation of L-alanine.

Galactose is not fermented by most dairy strains of the yoghurt bacterium *S. thermophilus*. This is a result of the absence of the enzyme galactokinase. The genes coding for galactokinase and for the other enzymes of the Leloir pathway are, however, present but are not transcribed. This is caused by isolated mutations in the *gal* promoter region [9]. Galactose-fermenting mutants have been isolated and can be constructed by repair of the promoter mutations. Using these strains, the manufacture of yoghurt without galactose has become possible.

## Vitamin production

A clear example of health-promoting activity by lactic acid bacteria is the ability to produce vitamins. Although most lactic acid bacteria are known as fastidious, and often vitamin-auxotrophic bacteria, there are some known exceptions. The yoghurt bacterium *S. thermophilus* produces the B-vitamin folic acid, which is subsequently used, for growth, by the other yoghurt bacterium *L. bulgaricus*. By selecting high folic acid-producing strains or by using relative high amounts of *S. thermophilus* compared to *L. bulgaricus*, yoghurt with increased folic acid contents should, in principal, be possible. Folic acid is an essential component in human nutrition, is conspicuously absent or present at too low levels in many modern food products and is specifically recommended in the diet of pregnant women.

*L. lactis* has also the ability to produce folic acid. This B-vitamin seems to specifically involved in the biosynthesis of purines and pyrimidines. Interestingly, specific folic acid production was increased by a factor twenty under conditions of reduced growth rates as can be induced in chemostats or by addition of antimicrobials [10]. Some of the genes responsible for biosynthesis of folic acid have been cloned and currently attempts are underway to influence, via controlled expression, the production level of folic acid and its molecular structure.

## Conclusions

Metabolic activity of lactic acid bacteria during milk fermentation leads to dairy products with clear added value compared to milk. In flavour, structure and in health aspects, an improved product is the result of fermentation by lactic acid bacteria. Most of these bacterial activities, however, do not reach maximal functionality during milk fermentation and are up for improvement. This can be reached, as demonstrated by the presented examples, by metabolic engineering.

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