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Review

Metabolic engineering approaches for lactic acid production

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Abstract

Lactic acid is an important product of industrial importance. Various approaches using traditional and modern biotechnological approaches have been tried to improve the lactic acid production. The lactic acid bacteria, yeast and fungal systems have been engineered to enhance the lactic acid production. The advent of biotechnology and recognition of industrial applications of lactic acid led to the efforts being focused on use of biotechnological tools to engineer lactic acid bacteria (LAB) and other systems for the production of lactic acid. The initial efforts in LAB genetic modifications were concentrated mostly to develop LAB with enhanced qualities for food grade applications, using traditional approaches. The spontaneous mutations were also attempted by using insertion sequence (IS) elements. The LAB subjected to genetic improvement have been used in dairy industry for flavour enhancement, resistance to bacteriophages, addition of nutritional components and stability and structure of end products. The controlled gene expression systems for industrial gram-positive bacteria with low G + C content have already been reported. However, with the recognition of polylactide as a biodegradable polymer, attempts were directed to reduce the cost of lactic acid production by genetically modifying the organism, by using various cheaply available agro-industrial residues and by process modifications to remove the lactic acid produced during the course of fermentation. The authors here have tried to briefly summaries the various approaches to metabolic engineering used for improving the lactic acid production and cost reduction.

Keywords: Lactic acid bacteria; Genetic modification; Metabolic engineering; Production; Agro-industrial residues

Contents

1. Introduction

Lactate is an important end product of bacterial fermentation of glucose and other carbohydrates (Fig. 1). The name of the acid was derived from the Latin word for milk (lac). Many species of bacteria form lactate but amount of lactate produced depends on the carbohydrate, conditions of growth, and the metabolic pathway. The traditional food related fermentations are known for long time but now only the metabolic pathways involved are understood so that we can use selected strains and controlled conditions for carrying out a particular fermentation. The lactic acid bacteria are important in other food fermentations also, because their metabolism is diverted away from glycolysis to produce desired flavour compounds (Fig. 1). Another important role

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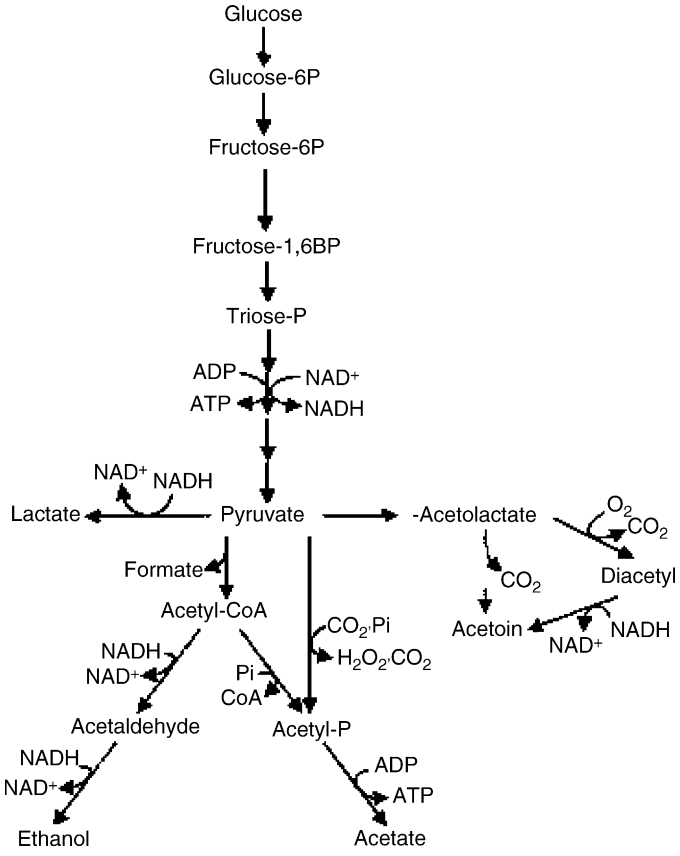

Fig. 1. Generalized pathway of glucose metabolism.

of lactic acid bacteria involves animal food. Traditionally, grass was preserved for animal food as hay, but now more of the nutrients are kept when herbage is converted to silage. Many studies have shown the beneficial effects on ruminant performance of feeding them with silages inoculated with lactic acid bacteria (LAB). These benefits might derive from probiotic effects [1,2]. These processes became possible only when fermentations by lactic acid bacteria were understood. The majority of LAB live free of any host species and are nonpathogenic. Some lactic acid bacteria are found in the guts of animals, where their activities affect the well-being of the hosts; others are associated with dental caries, and a few are pathogenic. Because lactic acid bacteria are generally regarded as safe (GRAS), hence, humans can use edible material preserved by the growth of lactic acid bacteria. Also, reducing conditions and low pH inhibit the growth of pathogenic and food spoilage bacteria, because growth of these organisms will cause food spoilage or may cause food borne diseases.

The importance of LAB and lactic acid production got a boost with the discovery of biodegradable polylactide polymer. Much of the growth is attributable to two emerging products: polylactic acid for biodegradable plastics [3] and the environmentally benign solvent ethyl lactate [4]. Due to this growing market for the biodegradable and renewable polymer polylactic acid, the world demand for lactic acid is increasing rapidly. The worldwide production of lactic acid, which is presently in excess of 100,000 metric tonnes per year, is rapidly increasing, mainly as a result of the growing market for polylactic acid [5,6]. It is expected that this biodegradable polymer, produced from renewable resources, will replace various petrochemical industry based polymers in applications ranging from packaging to fibers [7]. The major end product of LAB fermentation, lactate, has applications as a preservative, acidulant and flavouring agent in food industry, because of the tartness provided by lactate and also because lactate is generally regarded as safe (GRAS). Other uses of lactic acid, comprises applications in cosmetics, tanning industry and as intermediate in pharmaceutical processes [8].

2. Current problems in lactic acid production

The initial production technology of lactic acid fermentation was based on neutralization of produced acid with a suitable neutralizing agent. The major problems associated with lactic acid production by fermentation have been fastidious nutritional requirements of lactic acid bacteria, which add up to 35% of cost of production [9] and end product inhibition [10]. To remove the end product inhibition, attempts were directed to develop processes for removal of lactic acid from fermentation broth and various methods were reported for the purpose. These methods included cross flow filtration with cell recycling [11–13], electro dialysis [14–16], ion exchange resins [17,18] and extraction from fermentation broth [19–28]. The US patents 5,780,678 [23], 5,892,109 [25], 6,087,532 [26] 6,187,951 [27] and 6,472,559 [28], described the use of trialkyl amine extraction of lactic acid in presence of CO_2. The recovered carbonate or bicarbonate was recycled to the fermenter, also US patent 5,786,185 described use of solid phase polymer containing pyridine for lactic acid extraction from fermentation broth [24]. Oh et al., used cell recycle with repeated batch bioreactor for lactic acid production [9]. The US patent 6,475,759 described process for producing lactic acid, which includes incubating acid tolerant homofermentative lactic acid bacteria in nutrient medium to produce a fermentation broth with high levels of free lactic acid [29]. Several alternative substrates including agro-industrial residues have been tried to reduce the cost of production. Yun et al. [30] reported production of L-(+)-lactic acid from various carbohydrates using a newly isolated bacteria *Enterococcus faecalis* RKY1. Similarly Yun et al. [31] reported use of amylase treated rice and wheat bran hydrolysates for production of lactic acid without any other nutrient being added to the fermentation media. Wee et al. [32] reported the use of adapted *E. faecalis* RKY1 for lactic acid production from wood hydrolysate. Wee et al. [33] reported use of same culture for L-(+)-lactic acid production from sugar molasses and observed that lactic acid fermentation using molasses was significantly affected by yeast extract concentration. Woiciechowski et al. [34] used statistical experimental design to enhance the L-(+)-lactic acid production from steam-exploded wood hydrolysate using *Rhizopus oryzae*. Solid-state fermentation and agro-industrial residues have also being used for L-(+)-lactic acid production [35–39].

3. Approaches to genetic modification in LAB

With increasing demand for lactic acid and increasing concern over environmental impact of gypsum accumulation as a by-product of traditional fermentation, enhanced efforts for development of alternative technologies/methods are being made. The advent of biotechnology led to the efforts being focused on use of biotechnological tools to engineer for the production of lactic acid. The initial efforts in genetic modifications were concentrated mostly to develop LAB with enhanced qualities for food grade applications, using traditional approaches such as exposing to mutagenic agents such as ethyl methyl sulphonate, NNG, NTG and UV radiations. The spontaneous mutations were also attempted by using insertion sequence (IS) elements [40]. The LAB subjected to genetic improvement has been used in dairy industry for flavour enhancement, resistance to bacteriophages, addition of nutritional components and stability and structure of end products. The use of insertion sequence elements for deletion of *Lac Z* gene of *Lactobacillus bulgaricus* resulted in its limited fermentation of lactose. Hence, this strain could be used in yoghurt fermentation and limited lactose utilization prevents further souring of yoghurt [41]. The gene inactivation method based upon integration of insertion sequence (IS) elements into chromosome leads to altered gene expression pattern, which may give rise to a strain with enhanced qualities [42].

The genetic engineering approaches have been exploited in a big way for improvement of LAB. Table 1 gives brief account of work done for improvement of LAB using genetic modification. Inactivation of *aldB* gene encoding α-acetolactate decarboxylase had been used to improve the flavour and flavour stability of buttermilk [43]. Similarly spontaneous mutation was used for selection of a *Lactococcus lactis* strain that overproduced diacetyl, responsible for the butter flavour in many fresh dairy products [44]. The transformation of industrially important strains with phage resistant genes from other LAB has been used to generate new phage resistant strains [45]. The *α-Gal* structural genes from *Lactobacillus plantarum* ATCC8014 and from guar was cloned and expressed in *L. lactis* to develop a lactic acid bacterium capable of degrading non-digestible oligosaccharides (NDO) and used to study the problems associated with non-digestible oligosaccarides [46].

The five genes involved in folate biosynthesis pathway of *L. lactis* MG1363 were identified and designated as *folA*, *folB*, *folKE*, *folP* and *folC*. The gene *folKE* encodes the biprotein 2 amino 4-hydroxy 6 hydroxymethyl dihydropteridine pyrophophokinase and GTP cyclohydrolase I. These findings suggested feed back inhibition of reduced folate on folate biosynthesis [47]. Gene-specific genetic engineering has also been used to examine the genes of the rib operon's need to be over expressed in order to effect riboflavin overproduction in *L. lactis* subsp. *cremoris* strain NZ9000. Transcriptional regulation of the *L. lactis* riboflavin biosynthetic process as well as the analysis of roseoflavin-induced riboflavin-overproducing *L. lactis* isolates revealed the presence of both nucleotide replacements and deletions in the regulatory region of the *rib* operon [48]. These findings suggest that fermentation process can be used for producing foods containing increased levels of riboflavin and folate, negating the need for vitamin fortification.

The mannitol and lactic acid yields and productivities were practically unaffected by deletion of the *ldhD* gene of a mannitol producing *Lactobacillus fermentum*. The stepwise inactivation of the *ldh* genes involved gene replacement technique with deletion constructs removing a 0.4 kb fragment from the promoter and the 5′ end region of the *ldh* genes. In bioreactor cultivations, the single mutant GRL1030 produced mannitol and L-(+)-lactic acid as expected [49]. The engineering of *L. lactis* to a mannitol producing strain was carried out by disruption of two genes *mtlA* and *mtlF* by double-crossover recombination in strain *L. lactis* FI9630 (a food-grade lactate dehydrogenase-deficient strain derived from MG1363), yielding two mutant (*_ldh_mtlA* and *_ldh_mtlF*) strains having disrupted phosphoenolpyruvate mannitol phosphotransferase system. The new strains, FI10091 and FI10089, respectively, do not possess any selection marker and were proposed to be suitable for use in the food industry [50]. It was suggested that *ldh* deficient and the *mtlD* over expressing *L. lactis* was the most promising strain for mannitol production [51].

Attempts have also been made to study the regulatory mechanisms for metabolism and also role of regulatory sequences. The *L. lactis ccpA* gene, encoding the regulatory protein CcpA was identified and characterized. The *L. lactis ccpA* gene is constitutively transcribed from a promoter lacking a *cre* sequence. The functions of *ccpA* gene can be complemented, indicating conserved nature of gene. The

Table 1
Improvement of LAB using genetic modification

SN	Reference	Organism	Target	Outcome
1	Mollet et al. [41]	*Lactobacillus bulgaricus*	*lac Z*	Restricted lactose utilization
2	Swindell et al. [43]	*Lactococcus lactis*	*aldB* gene	Flavour and stability
3	Luesink et al. [52]	*L. lactis*	*ccpA* gene	Inhibition of catabolite repression of gal operon
4	Monnet et al. [44]	*L. lactis*	*aldB* and *ldh*	Overproduction of diacetyl
5	Le Blanc et al. [46]	*L. lactis*	α-Gal structural genes	Degradation of NDO
6	Sybesma et al. [47]	*L. lactis* NZ9000	*rib* operon	Production of folate
7	Burgess et al. [48]	*L. lactis* NZ9000	*rib* operon	Increased levels of riboflavin
8	Aarnikunnas et al. [49]	*Lactobacillus fermentum*	*ldhD*	Mannitol and L-(+)-lactic acid
9	Gaspar et al. [50]	*L. lactis*	*mtlA* and *mtlF*	Mannitol-production
10	Wisselink et al. [51]	*L. lactis*	*mtlD*	Mannitol production

disruption of the *ccpA* gene reduces the catabolite repression of the gal operon, which contains a cre site at the transcription start site and encodes enzymes involved in galactose catabolism. In contrast, CcpA activates the transcription of the *cre* containing promoter of the *las* operon, encoding the glycolytic enzymes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase [52]. Three isogenic strains of *L. lactis*, the parent strain *L. lactis* MG1363, a NOX^- strain harboring a deletion of the gene coding for H_2O-forming NADH oxidase, and a NOX^+ strain with the NADH oxidase activity enhanced by about 100-fold were used to study the effect of oxygen on glucose metabolism. (i) The fructose 1,6-bisphosphate (Fru-1, 6-P_2) level was lower under aerobic than under anaerobic conditions, and rate of Fru-1, 6-P_2 depletion was very high; (ii) the levels of 3-phosphoglycerate and phosphoenolpyruvate were considerably enhanced under aerobic conditions and significantly lower in the NOX^- strain; (iii) the glycolytic flux decreased in the presence of saturating levels of oxygen, but it was not altered in response to changes in the NADH oxidase activity. This indicated that fructose 1,6-bisphosphate (Fru-1, 6-P_2) has a role in glycolytic flux control and also glycolytic flux is affected by oxygen concentration but is not primarily determined by the level of NADH in the cell [53]. It has also been observed that the glycolytic flux was unchanged in the mutants overproducing glyceraldehyde 3-phospahte dehydrogenase (GAPDH). This suggested that the GAPDH activity has no control over the glycolytic flux at the wild-type enzyme level and that the enzyme is present in excess capacity by a factor of 3–4. Also the glycolytic flux in resting cells is even more insensitive to changes in the GAPDH activity because GAPDH was also present in large excess and had no control over the glycolytic flux [54]. Two-component signal-transduction machinery was proposed for transcription activation and production of several auto inducers found in LAB, which are predominantly bacteriocins or bacteriocin-like peptides. In the nisin auto regulation process in *L. lactis* the NisK protein acts as the sensor for nisin and the NisR protein as the response regulator, activating transcription of target genes. The *cis*-acting elements *nisA* and *nisF* are promoter fragments for NisR [55].

Attempts have also been made to completely sequence the genome of LAB so that information regarding metabolic pathways can be generated. The sequencing of the genome of the laboratory strain *L. lactis* IL1403 showed that the genome contained 2,365,589 base pairs (AE005176) and encodes for 2310 proteins, including 293 protein-coding genes belonging to six prophages and 43 insertion sequence (IS) elements. Non-random distribution of IS elements indicated that the chromosome of the sequenced strain may be a product of recent recombination between two closely related genomes. Analysis of sequence data showed that a complete set of late competence genes was also present, indicating the ability of *L. lactis* to undergo DNA transformation. Genomic sequence data reveals new possibilities for fermentation pathways and for aerobic respiration and also indicates a horizontal transfer of genetic information from *Lactococcus* to gram-negative enteric bacteria of *Salmonella-Escherichia* group [56]. Similarly, *L. plantarum* strain WCFS1 sequence was determined to be 3,308,274 bp and 3052 protein-coding genes were predicted [57]. The *L. plantarum* strain WCFS1 (NCIMB8826) was originally isolated from human saliva.

4. Lactate dehydrogenase

Garvie has given a beautiful account of lactate dehydrogenases present in different systems [58], however, to the best of our knowledge no subsequent efforts were made to account for the work done afterwards. Here, authors have tried to present a brief account of work done afterwards (Table 2) for studying lactate dehydrogenases from different sources.

The nucleotide sequence of 1.29 kb of *Streptococcus mutans* DNA that contained the promoter and protein-coding region of the gene *ldh* was determined and it was confirmed that *S. mutans* JH1000 contained a single copy of the lactate dehydrogenase gene [59]. The cloning of an allosteric *ldhL* gene of *Lactobacillus casei* ATCC 393 in *Escherichia coli* revealed that the open reading frame consisted of 981 bp, starting with a GTG codon and ending with a TAA codon. The sequences for the promoter, ribosome-binding site, and for a structure resembling a p-independent transcription terminator were also identified [60].

Similarly, gene encoding D-(−)-lactate dehydrogenase (*ldhD*) of *L. plantarum* with an inducible expression plasmid, in which the 5′-noncoding region of the gene was replaced with the *tac* promoter was expressed in *E. coli*. The comparison of the sequence of *ldhL* with *ldhD* including the *L. plantarum ldhL* showed no significant homology among them. However, the *ldhD* was homologous to *E. coli* D-3-

Table 2
Lactate dehydrogenase enzyme from different sources

SN	Reference	Gene	Source organism	Outcome
1	Duncan and Hillman [59]	*ldh*	*Streptococcus mutans* JH1000	Single copy gene
2	Kim et al. [60]	*ldhL*	*Lactobacillus casei* ATCC 393	981 bp ORF identification
3	Taguchi and Ohta [61]	*ldhD*	*Lactobacillus plantarum*	2-Hydroxyacid dehydrogenase
4	Llanos et al. [62]	*ldhL*	*L. lactis* subsp. *lactis*	Allosteric enzyme of 324 amino acids
5	Kochhar et al. [63]	*ldhD*	*Lactobacillus helveticus*	NAD (+)-dependent, 336 amino acids protein
6	Savijoki and Palva [64]	*ldhL*	*L. helveticus*	Non-allosteric enzyme of 323 amino acids
7	Weeks and Yuksel [65]	*ldhD* and *ldhL*	*Lactobacillus* sp. strain MONT4	1002 bp *ldhD* ORF coding for 334 amino acids, 963 bp *ldhL* ORF coding for 321 amino acids
8	Lee et al. [66]	*ldhL* and *ldhD*	*Lactobacillus* sp. RKY2	962 and 998 bp ORFs

phosphoglycerate dehydrogenase and *L. casei* D-2-hydroxyisocaproate dehydrogenase suggesting that D-lactate dehydrogenase was a member of a new family of 2-hydroxyacid dehydrogenases. These being distinct from L-lactate dehydrogenase and L-malate dehydrogenase, and also that the new family consisted of D-isomer-stereo specific enzymes. The D-lactate dehydrogenase is also having D-glycerate dehydrogenase activity, because of the conserved residues in this family appear to be the residues involved in the substrate binding and the catalytic reaction [61].

It has also been demonstrated that L-(+)-lactate dehydrogenase from *L. lactis* subsp. *lactis* was activated by fructose-1,6-bisphosphate. Plasmids containing *ldhL* conferred fructose-1, 6-bisphosphate activated L-(+)-lactate dehydrogenase activity on *E. coli* cells, and a promoter was introduced to express the cloned *ldh*. The nucleotide sequence of *ldh* predicted a chain length of 324 amino acids and a subunit molecular weight of 34,910 for the enzyme, after removal of the N-terminal methionine residue. *L. lactis* subsp. lactis *ldh* was suggested to be a product of posttranscriptional processing transcribed from a 4.1 kb transcript [62]. NAD (+)-dependent D-lactate dehydrogenase from *Lactobacillus helveticus* was purified and first 36 amino acid residues sequence was determined. The plasmid over expression showed that D-lactate dehydrogenase was greater than 60% total soluble cell protein and plasmids were stable in *E. coli*, compared to plasmids carrying the *L. bulgaricus* and *L. plantarum* genes. The entire nucleotide sequence of the *L. helveticus ldhD* gene was determined and used to deduce amino acid sequence. This indicated a polypeptide consisting of 336 amino acid residues, showing significant amino acid sequence similarity to D-2-hydroxy-acid dehydrogenases family. The physicochemical and catalytic properties of recombinant D-lactate dehydrogenase were identical to those of the wild-type enzyme, e.g. α 2 dimeric subunit structure, isoelectric pH, Km and Kcat for pyruvate and other 2-oxo-acid substrates. However the kinetic profiles of 2-oxo-acid substrates showed differences from that of L-lactate dehydrogenase, suggesting different mechanisms for substrate binding and specificity [63].

The *ldhL* gene with capacity to encode a protein of 323 amino acids (35.3 kDa) was isolated from *L. helveticus*. The amino acid sequence of *ldhL* showed a high degree of identity with *ldhL's* of other *Lactobacilli*. The highest identity (80.2%) was observed with the *L. casei ldhL*. The sizes and 5′ end analyses of *ldhL* transcripts showed that the *ldhL* gene of *L. helveticus* was a monocistronic transcriptional unit. The expression of *ldhL*, studied as a function of growth, revealed a high expression level at the logarithmic phase of growth. The *ldhL* ORF organization in *L. helveticus* showed two putative −10 regions without corresponding −35 regions and the *ldhL* transcripts to be derived from the −10 region closest to the initiation codon with presence of additional putative −10/−35 regions. These findings suggest that L-LDH of *L. helveticus* is a non-allosteric enzyme, and amino acid residues involved in allosteric regulation are not conserved. Although optimal reaction velocity was at pH 5.0, however, enzyme particularly at neutral pH showed a slight enhancement of activity in the presence of fructose 1,6-diphosphate [64].

Two lactate dehydrogenase (*ldh*) genes from *Lactobacillus* sp. strain MONT4 were cloned by complementation in *E. coli* DC1368 (*ldh pfl*) and were sequenced. The sequence analysis revealed a 1002 bp *ldhD* ORF potentially coding for 334 amino acids. While, a 963 bp *ldhL* ORF coding for 321 amino acids was identified as responsible for L-(+)-lactate dehydrogenase expression. The subcloning of the individual *ldh* genes and their Northern blot analyses indicated that the genes are monocistronic [65]. The open reading frames of *ldhL* and *ldhD* from *Lactobacillus* sp. RKY2 encoding L-(+) and D-(−)-lactate dehydrogenases were found to be 962 and 998 bp, respectively with both the proteins showing highest homology with lactate dehydrogenases from *L. plantarum* [66].

The recent findings suggest co-regulation of lactococcal lactate dehydrogenases (LDHs) at the substrate level by at least two mechanisms: the fructose-1, 6-bisphosphate/phosphate ratio and the NADH/NAD ratio. Among the *L. lactis* species, there are strains that are predominantly regulated by the first mechanism (e.g. strain 65.1) or by the second mechanism (e.g. strain NCDO 2118) [67].

5. Metabolic engineering for lactic acid production

A few attempts have been made to improve L-(+)-lactic acid production by metabolic engineering in lactic acid bacteria and also in fungal and yeasts systems. Table 3 presents a brief account of metabolic engineering approaches for lactic acid production.

The plasmid pSUW100 encoding the D-(−)-lactate dehydrogenase from *L. helveticus* CNRZ32 was designed for deletion studies and nucleotide sequence of the *ldhD* was determined. A putative 1014 bp *ldhD* reading frame encoding a polypeptide of 337 amino acid residues with a deduced molecular mass of 38 kDa was identified. The homology of this gene was detected with leuconostocs and pediococci genes but not with lactococci or *L. casei*. The *ldhD* negative derivative of *L. helveticus* CNRZ 32 showed production of only L-(+)-lactic acid. However, no significant difference in growth or total lactic acid production was observed between CNRZ32 and its *ldhD* mutant [68].

The cloning of a gene (designated *ldhL*) encoding L-(+)-lactate dehydrogenase from *L. plustanm* DG301and analysis of nucleotide sequence of the *ldhL* gene predicted a protein of 320 amino acids closely related to that of *Lactobacillus pentosus*. Introduction of multiple copies of gene in *L. plantarum* resulted in 13-fold increase in L-LDH activity. However, this failed to increase the production of L-(+)- and D-(−)-lactate. A stable chromosomal deletion in the *ldhL* gene of *L. plantarum* resulted in the absence of L-LDH activity and in exclusive production of the D isomer of lactate [69]. Similarly in *L. lactis*, when the copy number of the lac operon in which the *ldhL* gene was present was increased, it resulted in a slight increase in lactic acid production [70].

The pyruvate decarboxylase (*PDC*) deficient *Saccharomyces cerevisiae* strain used for expression of lactate

Table 3
Metabolic engineering approaches for lactic acid production

SN	Reference	Approach	Organism	Outcome	Yield/conc.
1	Bhowmik and Steele [68]	*ldhD* deletion	*L. helveticus* CNRZ32	No significant difference in total lactic acid	–
2	Ferain et al. [69]	*ldhL*	*L. plustanm* DG301	No increase in lactate production	15.6 ± 1.0 g/l lactate
3	Davidson et al. [70]	lac operon copy number increase	*L. lactis*	Slight increase in lactic acid production	–
4	Adachi et al. [71]	*ldh* cloning and pyruvate decarboxylase (*PDC*) deletion	*Saccharomyces cerevisiae*	Increased lactate yield	11.36 g/l
5	Porro et al. [72]	Bovine *ldhL* gene cloning	*K. lactis*	Improved yields of lactic acid	109 g/l
6	Nikkila et al. [73]	*ldhD* deletion	*L. helveticus* CNRZ32	Exclusive production of L-(+)-lactic acid	50.64 g/l
7	Bai et al. [77]	Random mutagenesis	*Rhizopus oryzae*	Improved lactic acid production	79.4 g/l
8	Skory [75]	*ldhA* from *R. oryzae*	*S. cerevisiae*	Lactic acid production	38 g/l
9	Skory [76]	*ldhA* constructs for expression	*R. oryzae*	Increase in lactic acid production in some constructs	72.8–76 g/l
10	van Maris et al. [78]	*PDC* deletion	*S. cerevisiae*	Homofermentative lactate producing strain	1.66 ± 0.04 mol of lactate/mol of glucose
11	Saitoh et al. [79]	Bovine *ldhL* expression	Wine yeast strain	L-(+)-lactate production	122 g/l

dehydrogenase showed increased lactate yield with suppressed ethanol production. The findings also suggested that out of three genes (*PDC1*, *PDC5* and *PDC6*) responsible for pyruvate decarboxylase, *PDC1* plays a key role [71]. Similarly, introduction of bovine L-(+)-lactate dehydrogenase gene (*ldh*) into a wild-type *Kluyveromyces lactis* yeast strain showed co-production of lactate and ethanol. Deletion of unique pyruvate decarboxylase gene led to further improvement of the strain and concentrations, productivities, and yields of lactic acid as high as 109 g/l, 0.91 g/l/h, and 1.19 mol/mole of glucose consumed, were obtained. It was also observed that the engineered strain produced organic acid at pH levels lower than those usual for bacterial processes [72].

The expression of *ldhD* and *ldhL* and production of D-(−)- and L-(+)-lactic acid were studied in *L. helveticus* CNRZ32. In order to develop a host for production of pure L-(+)-isomer of lactic acid, two *ldhD*-negative *L. helveticus* CNRZ32 strains were constructed using gene replacement. One of the strains was constructed by deleting the promoter region of the *ldhD* gene, and the other was constructed by replacing the structural gene of *ldhD* with an additional copy of the structural gene (*ldhL*) of the same species. The resulting strains were designated GRL86 and GRL89, respectively. In strain GRL89, the second copy of the *ldhL* structural gene was expressed under the *ldhD* promoter. The two *ldhD* negative strains produced only L-(+)-lactic acid in an amount equal to the total lactate produced by the wild type. The maximum L-LDH activity was found to be 53 and 93% higher in GRL86 and GRL89, respectively, than in the wild-type strain. The L-(+)-lactic acid production by GRL89 was optimized using statistical experimental design and response surface methodology. The temperature and pH optima were found to be 41 °C and pH 5.9. At low pH, when the growth and lactic acid production are uncoupled, strain GRL89 produced approximately 20% more lactic acid than GRL86 [73].

The *Rhizopus* fungal system has also been used for metabolic engineering studies. The lactate dehydrogenase enzyme from *R. oryzae* has been cloned and expressed in *E. coli* [74]. The L-LDH gene (*ldhA*) from the filamentous fungus *R. oryzae*, expressed under control of the *S. cerevisiae adh1* promoter and terminator was introduced into yeast system. The diploid isolate accumulated approximately 40% more lactic acid with a final concentration of 38 g lactic acid/l and a yield of 0.44 g lactic acid/g glucose. The optimal pH for lactic acid production by the diploid strain was pH 5. *S. cerevisiae* mutants with diminished pyruvate decarboxylase activity and mutants with disrupted alcohol dehydrogenase activity showed transformants with diminished ethanol production. However, the efficiency of lactic acid production also decreased [75]. Similarly, transformation of *R. oryzae* with *ldhA* constructs revealed that some constructs gave lactic acid more than control strain [76]. A L-(+)-lactic acid over-producing mutant, *R. oryzae* R1021, was isolated by mutagenizing the parent strain (*R. oryzae* R3017) with UV, diethyl sulfate (DES) and 60Co. Starting with a concentration of 120 g/l corn starch, mutant R1021 produced 79.4 g/l L-(+)-lactic acid after 60 h in flasks, 52% higher than that produced by the parent strain. The L-(+)-lactic acid purity was 99.05% by weight based on the amount of total lactic acid. The mutant R1021 was also morphologically different from the parent strain. The results of carbon flux analysis of the parent strain and mutants showed that the pyruvate node in the metabolic model was the principle and flexible node. The results show that the key steps of the pathways in parent strain where most carbon is lost from lactic acid formation are the reactions form pyruvate to acetyl-CoA, oxaloacetate and ethanol. Although the fractions of the carbon

from pyruvate to ethanol and acetyl-CoA were reduced in mutants, these two pathways are still the steps that need to be further targeted to reroute the pyruvate metabolism to improve lactic acid production [77].

The *S. cerevisiae* (CEN.PK background) was engineered to homofermentative lactate-producing yeast by deletion of the three genes encoding pyruvate decarboxylase and introduction of a heterologous lactate dehydrogenase (EC 1.1.1.27). Like all pyruvate decarboxylase-negative *S. cerevisiae* strains, the engineered strain required small amounts of acetate for the synthesis of cytosolic acetyl-coenzyme A. Exposure of aerobic glucose-limited chemostat cultures to excess glucose resulted in the immediate appearance of lactate as the major fermentation product. However, the engineered strain could not grow anaerobically, and lactate production was strongly stimulated by oxygen also under all conditions examined, lactate production by the engineered strain was slower than alcoholic fermentation by the wild type [78]. Similarly, transgenic wine yeast strain containing six copies of the bovine L-lactate dehydrogenase gene on the genome were constructed. The recombinant strain upon fermentation with cane juice media produced L-(+)-lactate to the tune of 122 g/l with an optical purity of 99.9% or higher [79]. The nicotinic acid (NiA) was the limiting factor for the lactate production from *S. cerevisiae* strain K1 expressing lactate dehydrogenase gene from *L. plantarum* [80].

6. Development of vector systems

Table 4 describes some of the vector systems used or metabolic engineering of LAB and *S. cerevisiae* and also studies regarding the search for strong promoters for expression of desired genes. Various attempts have been made to develop efficient vector systems for metabolic engineering of LAB. The commercially available vectors modified by introducing promoters and terminators and addition of genes of interest along with different selection markers suitable for the system in use [68,71,73,75,76,79] have been used for lactic acid production studies. Also, several improvements associated with the lactic acid bacteria involved indigenous plasmids [88,89] and vectors for different lactic acid bacteria were developed based on these indigenous plasmids [90,91]. The plasmids based systems rely on selection markers and strains for food grade applications are not supposed to contain the antibiotic selection markers or inducible gene expression systems. Hence, the markers based on natural properties of lactic acid bacteria [92] were an ideal choice for food related applications and several attempts were made to develop these vectors. A shuttle vector able to replicate in *E. coli* and in gram-positive bacteria containing a nisin-inducible promoter (P*nisA*) and genes encoding NisR and NisK was developed [84], for activating transcription from P*nis*A in the presence of nisin. *E. faecalis* pCF10 plasmid genes *prg*X, *prg*Y, and *prg*Z, encoding cytosolic, integral membrane, and cell surface proteins, respectively, were cloned downstream of P*nis*A and increased protein expression was observed in the presence of nisin [84]. Similarly, a shuttle vector able to replicate in both *Streptococcus* sp. and *E. coli* was developed by joining the *E. coli* plasmid pACYC184 (chloramphenicol and tetracycline resistance) to the streptococcal plasmid pGB305 (erythromycin resistance) [93]. Similarly, to screen for conditional promoters *alr* gene encoding alanine racemase was used in *L. plantarum* [85]. The D-cycloserine presence showed increased alr level requirement making it a quantitative factor to the primarily qualitative nature of the *alr* complementation screen. This study showed alanine racemase complementation screening

Table 4
Development of vector systems for metabolic engineering

Vector	Characteristics	Reference	Purpose
pJDC9	Em^r, *lacZ*, *strong* transcriptional terminators	Chen and Morrison [81]	Streptococcal gene cloning
pIL252	Em^r	Simon and Chopin [82]	Cloning in *Streptococcus lactis*
pLAB1301	Em^r, Cm^r shuttle vector *Escherichia coli – L. plantarum*	Josson et al. [83]	Broad host range vector development
pGIT005	Em^r, pJDC9 with a 1.65 kb *Alu* I fragment from *L. plantarum* DG301 containing *ldhL*	Ferain et al. [69]	Lactic acid production
pGIT032	Em^r, Cm^r, pLAB1301 with a 1.45 kbp *Kpn* I fragment from pGIT005 containing *ldhL*	Ferain et al. [69]	Lactic acid production
pSA3	Shuttle vector *E. coli*/*Streptococcus* by joining pACYC184 and pGB305, Cm^r, Tet^r, Em^r, Ts replicon	Bhowmik and Steele [68]	Lactic acid production
pADNS	*adh1* promoter, *ldhA*	Adachi et al. [71]	Lactic acid production
pEPL2	KIPDC1 promoter, URA3 marker	Porro et al. [72]	Lactic acid production
pKTH2154	*ldhD* construct in pSA3	Nikkila et al. [73]	Lactic acid production
pKTH2156	*ldhD* promoter and *ldhL* in pJDC9	Nikkila et al. [73]	Lactic acid production
pMSP3535VA	Km^r, pVA380-1 replicon, *nisRK* and P*nisA*	Bryan et al. [84]	Vector for Gram +ve system
pMSP3535	Em^r, pAMb1 and colE1 replicons, *nisRK*, P*nisA*	Bryan et al. [84]	Lactic acid production
pNZ7110	Amp^r, pUC18 derivative containing *cre* and T_{pepN}	Bron et al. [85]	*alr* as selection marker
p*ldhA*48X11	*ldhA* gene	Skory [75]	Lactic acid production
pNZ7119	Amp^r, pNZ7110 derivative with *cre* replaced by *L. lactis alr*	Bron et al. [86]	Conditional active promoters
pNZ7120	Em^r, pIL252 derivative containing *L. lactis alr* from pNZ7119	Bron et al. [86]	Conditional active promoters
pUI100	pJDC9, *ldhD-ldhL*, Em^r, P*nisA*, *nisR* and *nisK*	Weeks and Yuksel [65]	*ldh* cloning
yEPLDH	TPII promoter with *ldh* gene	van Maris et al. [78]	Lactic acid production
PZ*n zitR*	PZ*n* promoter, *zitR* repressor, *uspnuc*$^+$ and *lacL*$^+$*M*$^+$	Llull and Poquet [87]	Controlled expression system development

method can be used for the identification of promoters on their conditional or constitutive activity [85]. The regulatory signals (PZ*n* promoter and *zitR* repressor) of the *L. lactis zit* operon were used to develop a new expression system PZ*n zitR*. The expression system is induced upon Zn deficiency and can be easily manipulated for expression of desired genes by manipulating the environmental conditions [92].

7. Conclusions

Although efforts have been put in to metabolic engineering of LAB, but these were concentrated in food related applications. However, with increase in demand for PLA and environmental concerns regarding production of large amounts of gypsum, efforts are now directed to metabolically engineer LAB for lactic acid production. However, given the intrinsic problems of growth under anaerobic conditions and inability of LAB to produce lactic acid under low pH conditions, concerted efforts are required to develop LAB with tolerance to low pH/product inhibition and identification of suitable target sites for engineering to develop lactic acid hyper producing strains at low pH making recovery of lactate easier and resulting in less accumulation of gypsum. In yeast based system pyruvate carboxylase gene has been identified as a target for engineering, however to completely divert yeast metabolism for lactic acid production more such targets need to be identified. However development of tolerant LAB systems seems to be more economic in terms of production and overall yields.

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