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SHORT COMMUNICATION

Diacetyl and acetoin production from whey permeate using engineered *Lactobacillus casei*

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Abstract The capability of *Lactobacillus casei* to produce the flavor-related compounds diacetyl and acetoin from whey permeate has been examined by a metabolic engineering approach. An *L. casei* strain in which the *ilvBN* genes from *Lactococcus lactis*, encoding acetohydroxyacid synthase, were expressed from the lactose operon was mutated in the lactate dehydrogenase gene (*ldh*) and in the *pdhC* gene, which codes for the E2 subunit of the pyruvate dehydrogenase complex. The introduction of these mutations resulted in an increased capacity to synthesize diacetyl/acetoin from lactose in whey permeate (1,400 mg/l at pH 5.5). The results showed that *L. casei* can be manipulated to synthesize added-value metabolites from dairy industry by-products.

Keywords *Lactobacillus* · Metabolic engineering · Diacetyl · Acetoin · Whey permeate

Introduction

Lactic acid bacteria (LAB) can be used for the fermentation of several natural sources with the aim of obtaining lactic acid. In addition, they are amenable to genetic modifications that transform them into efficient living factories for the production of different metabolites [1]. Diacetyl and acetoin are important flavor compounds responsible for the buttery aroma of some food products and are used as additives in the food industry. Both compounds can be produced by LAB and are derived from α-acetolactate, but, while acetoin is formed by the activity of α-acetolactate decarboxylase, diacetyl results from a non-enzymatic oxidative decarboxylation (Fig. 1a). Normal glycolytic flux in these fermentative bacteria yields lactic acid from the intermediate pyruvate, with reoxidation of the NADH formed during glycolysis. Most metabolic engineering approaches followed to produce diacetyl/acetoin by fermentation have been developed in *Lactococcus lactis*. In this model LAB, strains have been constructed in which an important part of pyruvate flux is diverted towards the production of α-acetolactate that is ultimately converted to acetoin and diacetyl. This has been achieved through mutation of lactate dehydrogenase (*ldh*) or cofactor (NADH) engineering in combination with overexpression of α-acetolactate synthase (*als*) or acetohydroxyacid synthase (*ilvBN*) genes [4, 6, 9, 12]. As a result of it, the excess pyruvate is channelled to α-acetolactate. *ilvBN* genes have been expressed in *Lactobacillus casei*, an organism that shows marginal production of diacetyl/acetoin, resulting in increased diacetyl formation [2]. In an attempt to enhance diacetyl/acetoin production by raising the amount of pyruvate available for IlvBN, in this work we blocked pyruvate alternative pathways in *L. casei*. The strains obtained were tested in the fermentation of whey permeate, a by-product from the dairy industry that can be utilized for the production of added-value metabolites by LAB.

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Materials and methods

Bacterial strains and growth media

Lactobacillus casei strains BL23 (wild-type), BL180 (*lac::ilvBN*, [2]), BL249 (Δ*ldh*, [10]), BL250 (*lac::ilvBN*

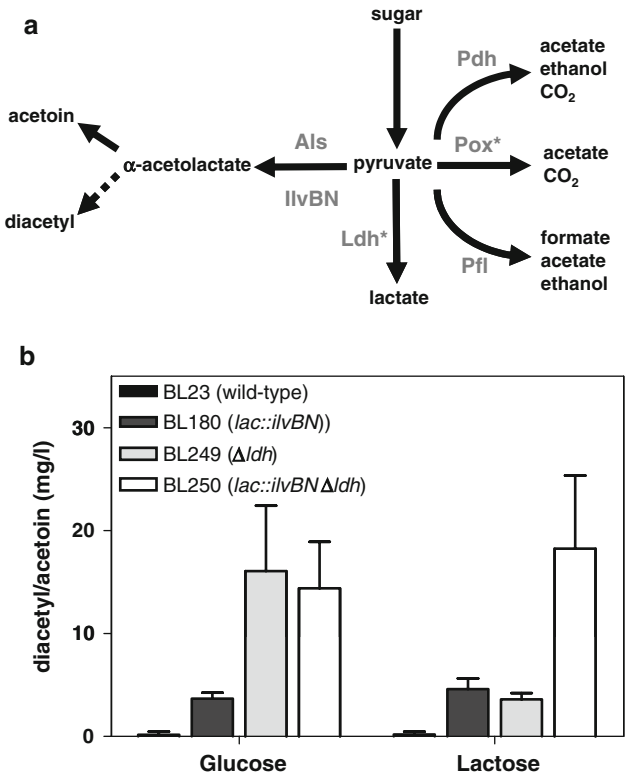

Fig. 1 **a** Alternative routes for pyruvate metabolism and the different end metabolites resulting from each pathway and **b** the effect of *ldh* mutation and/or IlvBN production in diacetyl/acetoin synthesis in *L. casei* resting cells. The means and standard deviations of three experiments are shown. *Als* α-acetolactate synthase, *IlvBN* acetohydroxy-acid synthase, *Ldh* lactate dehydrogenase, *Pdh* pyruvate dehydrogenase complex, *Pox* pyruvate oxidase, *Pfl* pyruvate–formate–lyase. *Asterisks* indicate activities that have several encoding genes in *L. casei* BL23

Δ*ldh*) and BL313 (*lac::ilvBN* Δ*ldh pdhC*) were grown in MRS medium at 37°C under static conditions. For fermentation experiments, dehydrated whey permeate was reconstituted at 60 g/l in distilled water. This substrate was treated with 100 µl/l of Flavourzyme™ (a peptidase enzyme mix from Novozymes that increased the amount of free amino acids) for 3 h at 50°C, then yeast extract was added to 0.5% (w/v), and the solution was autoclaved. *Escherichia coli* DH5α was used as cloning host, and it was grown in LB medium at 37°C under agitation. Ampicillin was used at 100 µg/ml for *E. coli*, and erythromycin was used at 5 µg/ml for *L. casei*. Solid medium was prepared by adding 1.8% (w/v) agar.

Strains construction

Lactobacillus casei BL180 was transformed with the integrative plasmid pRVΔldh1, a pRV300 [5] derivative that contains upstream and downstream fused sequences from the *L. casei ldh* gene [10]. Strains carrying integration of the plasmid by single cross-over were selected on plates containing erythromycin. One of them was grown for 200 generations in liquid medium in the absence of antibiotic, plated on MRS and replica-plated on MRS plus erythromycin. Among erythromycin-sensitive clones, one was selected in which a second recombination led to a complete deletion of *ldh* as confirmed by PCR [BL250 (*lac*::*ilvBN* Δ*ldh*)]. The *L. casei pdhC* gene (LCABL_15380) encoding the E2, dihydrolipoamide acetyltransferase, component of Pdh was amplified by PCR from BL23 strain with the oligonucleotide pair (5′-GGCTTTTGAATTCAAACTG-3′/5′-GGATCTGCCAACAATTTAT-3′). The PCR product was digested with *Eco*RI and *Kpn*I, and a 669-bp fragment cloned into pRV300 vector digested with the same enzymes, giving pRVpdhC. This plasmid was transformed into strain BL250, and clones carrying the plasmid integrated by single cross-over were selected on MRS plates plus erythromycin and checked by PCR analysis. Integration of pRVpdhC at the *pdhC* locus led to strains with a disruption of the gene. One of them was selected and named BL313 (*lac::ilvBN* Δ*ldh pdhC*).

Fermentation in resting cells

Lactobacillus casei strains were grown in MRS basal medium containing 0.5% lactose until an OD 550 nm of 0.8–0.9. Cells were washed twice with 100 mM sodium phosphate buffer pH 6.8 and resuspended to an OD 550 nm of 1 in the same buffer containing 1%(w/v) of either glucose or lactose. After 1 h of incubation at 37°C diacetyl/acetoin was determined in the supernatants.

Batch and fed-batch fermentations of whey permeate

Lactobacillus casei cells were grown in 1 l of whey permeate (pre-treated with Flavourzyme™) plus 0.5% yeast extract at 37°C in a 2-l water jacketed reactor (UniVessel, Sartorius) with stirring at 100 rpm. The reactor was inoculated at an initial OD 550 nm of 0.05 with cells grown in the same medium. The system was coupled to a Biostat B controller (Braun Biotech International), and constant pH (4.5, 5.5, 6.5 and 7.5) was maintained by addition of 1 M NaOH and 1 M HCl. Batch experiments proceeded for more than 150 h except for conditions where growth and production clearly reached a plateau at shorter times (i.e., pH 4.5). For fed-batch cultures, cells were grown until maximal diacetyl/acetoin production was reached, and 0.5% yeast extract, whey permeate or whey permeate plus 0.5% yeast extract was added at a flow rate of 5.6 ml/h.

Sugar and metabolite determination

Lactose and organic acids concentrations in whey permeate were determined by HPLC with a Jasco PU-2080Plus

system coupled to an UV detector (210 nm) or a refractive index detector (Jasco RI-2031Plus). Centrifuged samples were filtered (Millex-GV 0.22 µm filters, Millipore), applied to Rezex ROA-Organic Acid and Rezex RCM-Monosaccharide columns and separated in isocratic mode with 0.1% H_3PO_4, pH 2.4 or water as solvents, respectively. Diacetyl and acetoin were measured by a colorimetric reaction as previously described [13]. One milliliter of whey diluted samples (1:10–1:100 in distilled water) was mixed with 200 µl of 0.5% creatine and 200 µl of α-naphthol (5% in 2.5 N NaOH). The optical density was measured at 535 nm after 10 min and 1 h. This method primarily detects diacetyl, but acetoin is oxidized to diacetyl in the reaction solution at long incubation times. Freshly prepared dilutions of diacetyl in water were used for standard curves.

Results and discussion

Production of diacetyl/acetoin by resting cells

The main lactate dehydrogenase (*ldh*) gene was deleted in *L. casei* BL180. In this strain the *ilvBN* genes, encoding α-acetohydroxyacid synthase from *L. lactis*, are inserted at the *lac* locus, being therefore inducible by growth on lactose [2]. In resting cells pre-grown on lactose, the amount of diacetyl/acetoin produced from glucose or lactose was 21-fold higher in BL180 than in the wild-type strain (Fig. 1b). Introduction of an *ldh* deletion [BL250 (*lac::ilvBN* Δ*ldh*)] resulted in an increase in diacetyl/acetoin synthesis from glucose (84-fold), but a similar effect was also observed in the BL249 strain carrying only a single *ldh* deletion. However, bacterial cells of the BL249 strain exposed to lactose produced comparable amounts of diacetyl/acetoin to BL180 strain, whereas a fourfold increase in production was detected in BL250 strain. The already elevated production of diacetyl/acetoin in the single *ldh* mutant indicated the presence of endogenous α-acetolactate synthase activity. However, this production was dependent on the sugar used, being lower for lactose. No obvious explanation was found for this fact, and we decided to choose BL250 to further improve diacetyl/acetoin production from whey permeate, where lactose is the main fermentable sugar.

Construction of *L. casei* mutants affected in genes involved in pyruvate metabolism

Besides Ldh, three other activities (pyruvate oxidase, Pox; pyruvate–formate–lyase, Pfl and the pyruvate dehydrogenase complex, Pdh) are responsible for alternative pyruvate metabolic pathways (Fig. 1a). Genes encoding these enzymes were identified in the *L. casei* BL23 genomic sequence (GenBank FM177140). *L. casei* BL23 possesses three genes encoding putative Pox (*poxB1*, LCABL_20020; *poxB2*, LCABL_ 23230 and *poxB3*, LCABL_05020) whose products display 52, 49 and 39% identity, respectively, to the characterized Pox from *Lactobacillus plantarum* [7]. This fact indicates that redundant Pox activities may exist, and therefore the construction of a Pox-deficient mutant could involve difficulties. In fact, mutation of either *poxB1* or *poxB2* in BL250 did not show an effect on diacetyl/acetoin production (data not shown). The LCABL_16340 gene codes for a Pfl enzyme. This activity is only relevant under anaerobiosis, as trace amounts of oxygen irreversibly inactivate the enzyme. In fact, no formate production was detected under the fermentation conditions used in this study (data not shown). The Pdh complex consists of four subunits (E1α, E1β, E2 and E3) encoding pyruvate dehydrogenase, dihydrolipoamide acetyltransferase and dihydrolipoyl dehydrogenase activities, catalyzing the conversion of pyruvate to acetyl-CoA and CO_2. Similar to other bacteria, in *L. casei* BL23 the four Pdh encoding genes, *pdhABCD* (LCABL_15360 to LCABL_15390), are clustered in an operon. The *pdhC* gene was disrupted in *L. casei* BL250, giving BL313 (*lac::ilvBN* Δ*ldh pdhC*), which was affected in diacetyl/acetoin production (see below).

Batch and fed-batch fermentation of whey permeate

Whey permeate batch fermentations were carried out with BL250 and BL313 strains at different pHs. Growth of the strains in whey permeate was slow (more than 50 h to reach maximal OD) and displayed differences between the strains (Fig. 2). The best growth was achieved at pH 5.5 for both BL250 and BL313 strains (Fig. 2a, b), but a highest diacetyl/acetoin production was detected at pH 6.5 for BL250 and pH 5.5 for BL313 (Fig. 2c, d). Growth and metabolite formation in BL250 at pH 7.5 was severely affected, and therefore this pH was not tested with BL313 strain. Interestingly, strain BL313 could grow normally only at pH 5.5, suggesting that a functional Pdh may play a role in the physiology of pH homeostasis in *L. casei*. Product formation correlated with the growth profile (except for BL250 at pH 7.5, where no production was observed) with maximal concentrations at the beginning of the stationary phase. Lactose was never totally consumed and dropped to a minimum of 60 mM in BL250 at pH 6.5, while in BL313 final concentrations were always higher than 100 mM. Slow growth and lack of total lactose consumption may be a consequence of the integration of *ilvBN* at the *lac* locus, which may lead to detrimental effects on the expression of lactose utilizing genes or in the stability of the *lac* mRNAs [2]. This does not seem to be the general situation, because an *L. casei* strain with a sorbitol-6-P dehydrogenase gene

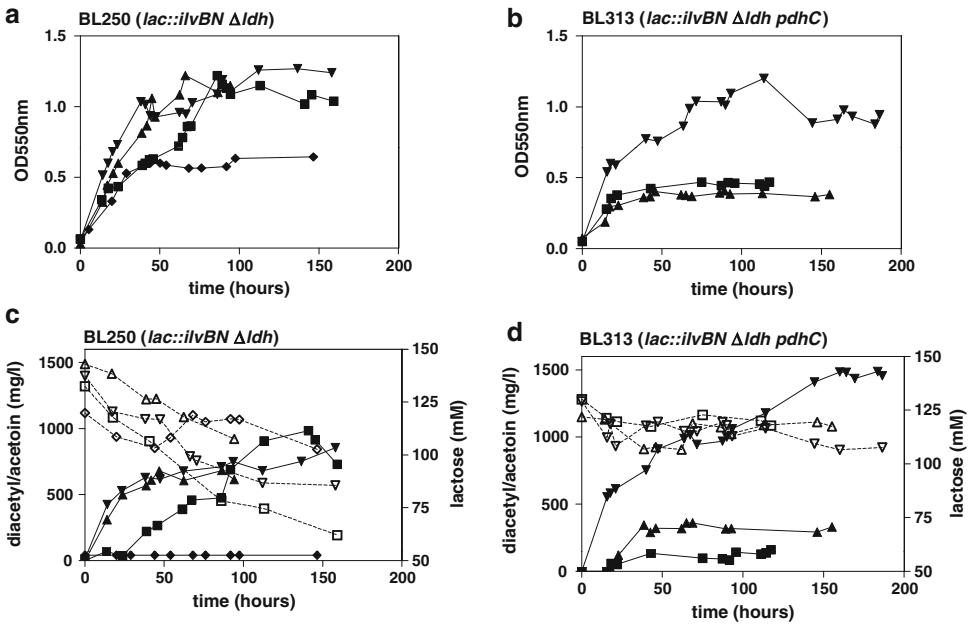

Fig. 2 Batch fermentations. Graphs showing growth (*closed symbols*), lactose consumption (*open symbols*) and diacetyl/acetoin production (*closed symbols*) of BL250 (**a** and **c**) and BL313 (**b** and **d**) strains in whey permeate at different pH 4.5 (*closed triangle*), 5.5 (*closed inverted triangle*), 6.5 (*closed square*) and 7.5 (*closed diamond*). Two independent experiments were done, and a representative is shown

integrated at the same position in an *ldh* background [8] is able to completely use lactose from whey permeate (unpublished observations). Total amount of diacetyl/acetoin produced by BL313 at pH 5.5 was higher than that produced by BL250 under optimal conditions (1,400 and 1,000 mg/l, respectively). In order to improve the amount of diacetyl/acetoin produced, a fed-batch assay was designed where, to prevent nutrient limitation, whey permeate, yeast extract or whey permeate plus yeast extract was added after diacetyl/acetoin production reached its maximum. These assays were initially carried out with BL250. Fed-batch experiments showed that in BL250 strain diacetyl/acetoin production could only be improved (1,200 mg/l after more than 300 h of fermentation, Fig. 3) by adding complete fresh medium (whey permeate plus yeast extract). Therefore, similar fed-batch experiments were carried out with BL313 strain by the addition of whey permeate plus yeast extract. However, no increase in diacetyl/acetoin concentration was seen under these conditions (Fig. 3). This strain already produced 1,400 mg/l after 150 h of growth at pH 5.5. The lack of diacetyl/acetoin increase in fed-batch suggests that the high amounts of formed product might be inhibitory. In fact, diacetyl is considered an antimicrobial compound due to the presence of carbonyl reactive groups. Pdh activity is inhibited by high NADH concentrations [11], which are likely to occur in cells with an *ldh* background. Nevertheless, it seems that Pdh is active in *L. casei* under the tested growth conditions, as we showed that a *pdhC* disruption affected growth and metabolite formation. Similarly, *L. lactis* mutants obtained by ethyl methanesulfonate treatment, which displayed reduced Pdh activity, showed an increase in acetoin production [3]. The amount of product obtained

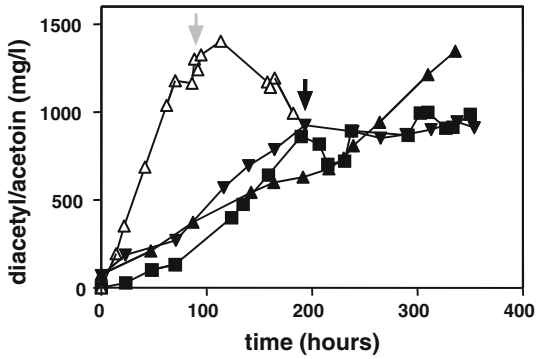

Fig. 3 Fed-batch fermentations. Diacetyl/acetoin production of BL250 strain at pH 6.5 (*closed symbols*) and BL313 strain at pH 5.5 (*open symbols*). A *black arrow* indicates the time of the start of a fed batch by addition of 0.5% yeast extract (*closed square*), whey permeate (*closed inverted triangle*) or whey permeate plus 0.5% yeast extract (*closed triangle*) in BL250 strain. A *grey arrow* indicates the start of the fed batch [addition of whey permeate plus 0.5% yeast extract (*open triangle*)] in BL313 strain. Two independent experiments were done, and a representative is shown

in this work (16 mM for BL313 strain) was still far from that obtained in other engineered *L. lactis*. In this bacterium, *ldh* deletion in combination with α-acetolactate synthase overexpression results in formation of 36 mM acetoin from M17 medium plus lactose [9]. However, the work reported here shows that a residual natural source, such as milk whey permeate, can be efficiently fermented, yielding a 35% conversion of pyruvate (16 mM product from 23 mM lactose utilized). This encourages the use of *L. casei* BL313 through complementary strategies leading to optimized diacetyl/acetoin production. These should address the likely diacetyl/acetoin inhibition, overexpression

of *ilvBN* or *als* from different chromosomal locations, improvement of lactose uptake and mutation of additional *ldh* genes.

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