


Cloning and Overexpression of the *als*, *pflA*, and *adhB* Genes in *Streptococcus thermophilus* and Their Effects on Metabolite Formation

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Abstract *Streptococcus thermophilus* is a lactic acid bacterium and used as starter culture in the dairy industry, mainly in the manufacture of yoghurt, with *Lactobacillus delbrueckii* subsp. *bulgaricus*. It produces lactic acid as a major fermentation end product and some carbonyl compounds through sugar metabolism. The level of metabolites could be improved using molecular biotechnology. The genes of *als*, encoding α -acetolactate synthase (Als), the *pflA*, encoding pyruvate-formate lyase activating enzyme (PflA), and the *adhB* which encodes alcohol dehydrogenase (AdhB) of *S. thermophilus* NCFB2393 strain were amplified by polymerase chain reaction and separately cloned into the overexpression vector pNZ276 under the control of the *lacA* promoter. The strains were transformed individually with the constructed plasmids. Their abilities to generate important metabolites such as pyruvate, lactate, formate, acetaldehyde, acetoin, ethanol, and 2,3-butanediol in LM17 medium were analyzed using high-performance liquid chromatography. High level of 2,3-butanediol was obtained by overexpressing the *als* gene. The level of

formate increased slightly by overexpressing the *pflA* gene. The overexpression of the *adhB* gene, on the other hand, resulted in a significant increase in the ethanol level.

Keywords Gene overexpression · *als* · *pflA* · *adhB* · Metabolic pathways

Introduction

Streptococcus thermophilus is an important lactic acid bacterium in the dairy industry and has long been used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* as a starter culture for yoghurt production. It has also been used in the manufacture of certain types of cheese. The main role of *S. thermophilus* in fermentation is to acidify the environment through the conversion of lactose into lactic acid and to produce a variety of compounds that contribute to flavor and texture of the final product. *S. thermophilus* is a “generally recognized as safe” (GRAS) species and it is the second important industrial strain following *Lactococcus lactis*. *S. thermophilus* belongs to the group of the thermophilic lactic acid bacteria and it is used not only by itself but also in combination with other species in dairy fermentations [16]. *S. thermophilus* can metabolize five different sugars such as lactose, sucrose, glucose, galactose, and fructose [34] and can degrade all amino acids [27]. Therefore, *S. thermophilus* strains show better growth than *L. bulgaricus* strains when grown in pure culture. However, the *S. thermophilus*—*L. bulgaricus* cooperation stimulates the growth of *S. thermophilus* strain [11].

Ten *S. thermophilus* strain genomes have been sequenced and published until 2015. The genomes and their four completed direct submission sequences are present in the NCBI

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Genome database [7, 18, 20, 21, 31–33, 36]. Most of the transport proteins and the proteins that have a role in energy metabolism related to carbohydrate degradation, uptake, and fermentation are pseudogenes. *S. thermophilus* is an obligate homolactic bacterium and produces L-lactate as main end products. The production of low-level additional fermentation end products, such as formate, acetoin, diacetyl, acetaldehyde, α -acetolactate, and acetate by *S. thermophilus* has also been reported [26, 28]. L-lactate dehydrogenase (Ldh), pyruvate-formate lyase (Pfl), pyruvate-formate lyase activating enzyme (PflA), and the α -acetolactate (Als) enzyme are known to be active in *S. thermophilus* pyruvate pathways. *S. thermophilus* has alternative routes other than lactate dehydrogenase for the production of certain metabolites; the phosphotransacetylase (EutD) and acetate kinase (AckA) pathways for acetate production, the α -acetolactate decarboxylase (AldB) route for acetoin production, and alcohol dehydrogenase (AdhB) for ethanol production [16]. Alcohol/acetaldehyde dehydrogenase genes (ψ *adhA* and ψ *adhE*) and the diacetyl/acetoin reductase genes (ψ *butA*, Ψ *str0909*, Ψ *str0910*) were determined as pseudogenes in completed genomes sequences (Fig. 1).

Inactivation of the *ldh* gene was attempted to change the functionality of the pyruvate dissipating pathways and for the implication of its role in the formation of aromatic products. However, inactivation of *ldh* gene was unsuccessful due to the absence of an alternative route for NAD^+

regeneration from pyruvate as shown by the reconstruction of pyruvate metabolism in *S. thermophilus* [16]. If the genes coding an enzyme involved in conversion of pyruvate to aromatic compounds can be overexpressed, the production of desired metabolites will increase. In this study, we describe a metabolic engineering approach to achieve high levels of enzyme production. For this purpose, we amplified the *als*, *pflA*, and *adhB* genes of *S. thermophilus* NCFB 2393 strain by PCR, cloned them into overexpression vector pNZ276, and created three new transformant plasmids. Effects of constructed plasmids on metabolite production were detected in aerobic conditions.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *S. thermophilus* NCFB 2393 [2] strains were routinely cultured in M17 medium supplemented with 1 % (w/v) lactose (LM17 medium) at 42 °C. *Escherichia coli* strains were grown on L broth or L agar [35] in a shaking incubator at 37 °C. Agar plate media contained 1.5 % (w/v) Bacto agar and antibiotic selection was used when appropriate for *E. coli*, 100 $\mu\text{g}/\text{mL}$ ampicillin, 15 $\mu\text{g}/\text{mL}$ of chloramphenicol and 200 $\mu\text{g}/\text{mL}$ of streptomycin,

Fig. 1 Pyruvate metabolic pathway of *Streptococcus thermophilus*. Existence of the genes in all completed genomes was searched. *ldh* L-lactate dehydrogenase, *pfl/pflA* pyruvate-formate lyase/pyruvate-formate lyase activating enzyme, *als* α -acetolactate synthase, *aldB* α -acetolactate decarboxylase, *eutD* phosphotransacetylase, *ackA* acetate kinase, *gapN* NADP-dependent glyceraldehyde-3-phosphate, *butA*, *str0909* and *str0910* acetoin reductase, *nox* NADH oxidase, *acoABL* acetoin dehydrogenase complex, *glyA* serine hydroxymethyltransferase, *adhB*, *str0880*, *str0881*, *str0882* alcohol dehydrogenase, Ψ *str1879-1884* aldehyde dehydrogenase, *Str_1034-1035* pyruvate dehydrogenase, and *pdc* pyruvate decarboxylase are present in *Z. mobilis*. ψ : pseudogenes

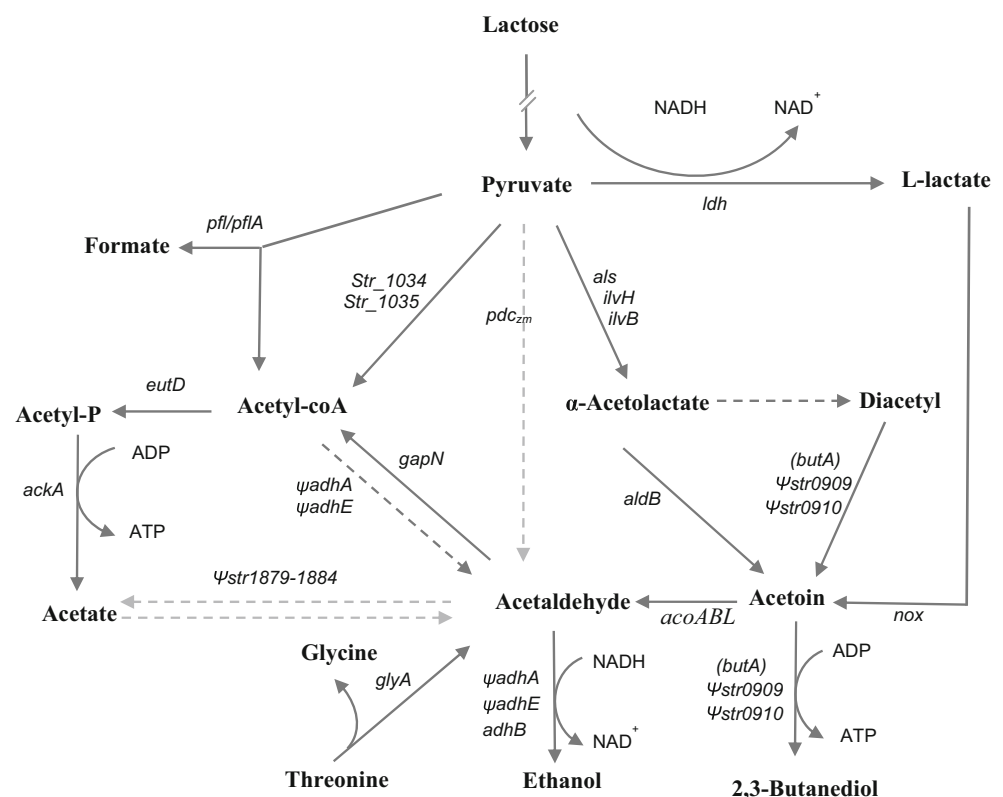


Table 1 Bacterial strains and plasmids used and constructed in this study

Strain/plasmid	Genetic description	Reference/source
<i>E. coli</i> strains		
MC1022	Plasmid-free strain, Str ^r	[9]
E-MMG121	MC1022/pMMG121, Amp ^r	This study
E-MMG122	MC1022/pMMG122, Amp ^r	This study
E-MMG124	MC1022/pMMG124, Amp ^r	This study
E-MMG131	MC1022/pMMG131, Cm ^r	This study
E-MMG132	MC1022/pMMG132, Cm ^r	This study
E-MMG134	MC1022/pMMG134, Cm ^r	This study
<i>S. thermophilus</i> strains		
NCFB2393 (FI8894)	Plasmid free strain	[2]
S-MMG131	NCFB2393/pMMG131, Cm ^r	This study
S-MMG132	NCFB2393/pMMG132, Cm ^r	This study
S-MMG134	NCFB2393/pMMG134, Cm ^r	This study
Used and constructed plasmids		
pJET1.2	General PCR cloning vector, Amp ^r	Fermentas
pNZ276	6113 bps, overexpression vector, Cm ^r	NIZO food research
p121	Derivative of pJET1.2 containing <i>AccI/XhoI</i> the <i>als</i> gene, Amp ^r	This study
p122	Derivative of pJET1.2 containing <i>AccI/XhoI</i> the <i>pflA</i> gene, Amp ^r	This study
p124	Derivative of pJET1.2 containing <i>PstI/XhoI</i> the <i>adhB</i> gene, Amp ^r	This study
p131	Derivative of pNZ276 containing <i>AccI/XhoI</i> <i>als</i> fragment, Cm ^r	This study
p132	Derivative of pNZ276 containing <i>AccI/XhoI</i> <i>pflA</i> fragment, Cm ^r	This study
p134	Derivative of pNZ276 containing <i>PstI-XhoI</i> <i>adhB</i> fragment, Cm ^r	This study

Amp^r ampicillin resistance, *Cm^r* chloramphenicol resistance, *Str^r* streptomycin resistance

and for *S. thermophilus* NCFB 2393 5 µg/mL of chloramphenicol.

Molecular Cloning Techniques

Plasmid DNA isolation, manipulation of DNA, and transformation of *E. coli* strains with recombinant plasmids were carried out as described previously [1, 19]. Restriction enzymes and other DNA-modifying enzymes from various sources were used according to the suppliers' recommendations. Constructed plasmids were introduced into *E. coli* by transformation using calcium chloride method, and introduced into *S. thermophilus* by electroporation according to Mollet et al. method [24]. Transformant colonies were selected on LB and LM17 agar plates containing appropriate antibiotics, respectively. Chromosomal DNA was isolated from *S. thermophilus* NCFB 2393 [2] strain according to the procedure reported by Vos et al. [35].

The *als*, *pflA*, and *adhB* genes were amplified by PCR. In order to clone the *als*, *pflA*, and *adhB* genes, the published *S. thermophilus* LMG18311 complete genome sequence (Accession no: NC006448) [7] was used to

design the primers. Primers were designed for amplification of the entire *als*, *pflA*, and *adh* genes including ribosome binding sites. Oligonucleotides were designed and obtained from Iontek (Istanbul, Turkey). Appropriate restriction sequence was added to each primer to insert into the overexpression vector pNZ276. The *als* gene fragment primers were engineered to contain *AccIII* and *XhoI* restriction sites in AlsF3 (5'-gcgaaatccGGAAAGTTGGCTCTACCATTTTA-3') and AlsR2 (5'-tatccgctc-gagTAGTAAAATTCATCT GGCAA-3') primers, respectively. The *als* amplification gave 1743 bp length DNA, including open reading frame and ribosome binding site regions. *AccIII* and *XhoI* restriction endonuclease sites containing PflAF (5'-tgcttcggaTGTA AAAATCGTTT TTCAAG-3') and PflAR (5'-gcgaaactcgagTTAATCCAA GGTTTTAACC-3') primers were used to amplify 841 bp length *pflA* gene. The *adhB* gene fragment (1078 bp length) was amplified using *PstI* and *XhoI* restriction sites added AdhBF (5'-gcgaaactcgagACTATAACTAAAGAAAT AG-3') and AdhBR (5'-tatccgctc-gagCTAAT CTAAAA CAATCA-3') primers.

The polymerase chain reaction (PCR) was performed in a DNA thermal cycler with the following thermal cycling

program: initial cycle, 2 min at 94 °C; next 30 cycles, 1 min at 94 °C; 1 min at annealing temperature; and 2 min at 72 °C. Different annealing temperatures (60, 68, and 55 °C) were applied for the amplification of the *als*, *pflA*, and *adhB* gene fragments, respectively. Colony PCR for routine screening of recombinant clones was performed after 5 min at 95 °C pre-denaturation step. PCR was done in 40 µL of reaction mixture containing 1 µL (20 pmol) forward primer, 1 µL (20 pmol) reverse primer, 1 µL dNTP (1 mM), 4 µL buffer (NH₄)₂SO₄ (10X), 1,6 µL MgCl₂, 1 µL DNA polymerase (5 µ/µL) [25 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT ve %50 (v/v) glycerol], 1 µL template DNA (~400 ng/mL), abd 29.4 µL dH₂O.

Fragments generated for the construction of vectors were amplified with Fermentas proof-reading DNA polymerase (Thermo Scientific, Turkey) and cloned into pJET1.2/blunt Cloning vector using the original CloneJET™ PCR Cloning kit (Thermo Scientific, Turkey) before nucleotide sequence confirmation. For routine PCR screening of recombinant clones, Taq DNA polymerase (Thermo Scientific, Turkey) was used. Ligation reactions were performed according to Sambrook et al. [29]. The nucleotide sequences of PCR-generated fragments were confirmed on purified plasmid DNA with an Applied Biosystems DNA sequencer (model 3130xl). DNA sequencing reactions were conducted using the DNA sequencing kit (ABI BigDye®) supplied by Applied Biosystems. pJET1.2 forward and pJET1.2 reverse primers were used with the DNA sequencing kit. The results of sequencing were analyzed using ChromasPro and Clone Manager 9 program.

One mL overnight culture was centrifuged at 14,000×g for 5 min and then resulting supernatant was filtered and transferred into a fresh eppendorf tube. The sample was then analyzed by HPLC or frozen in a dry ice/ethanol bath, and stored at –20 °C until analysis. HPLC analysis was carried out by diluting the sample onefold in 0.5 % meta-phosphoric acid, and injecting 20 µL of it onto the HPLC system. The separation was performed on a Capcell Pak® 5 µm C18 MG column (150 × 4.6 mm) at 30 °C. A Shimadzu Prominence HPLC apparatus (Shimadzu, Kyoto, Japan) equipped with a PD-M20A diode array detector and two binary gradient pumps (Shimadzu LC-10AT), autosampler (SIL 20AC), column oven (CTO-20AC), and a communication bus module (CBM-20A) with valve unit FCV-11AL was used. Standards of all metabolites were purchased from Sigma-Aldrich (Munich, Germany). The mobile phase consisted of meta-phosphoric acid and HPLC grade water. The HPLC system was calibrated using standards for sodium pyruvate (10 mg/mL), sodium lactate (10 mg/mL), sodium formate (10 mg/mL), acetoin (10 mg/mL), acetaldehyde (10 mg/mL), ethanol (10 mg/mL), and 2,3-butanediol (10 mg/mL) prepared in LM17 broth background. Quantification of the peak height

was performed using a PC1000 chromatographic data analysis system (Thermo Separation Products). Average of each metabolite was calculated from the results of three independent analyses.

Results

Construction of Overexpression Vectors Containing the *als*, *pflA*, and *adhB* Genes Under Control of the *lacA* Promoter

The genome sequence showed that the *als*, *pflA*, and *adhB* genes were transcribed mRNA in *S. thermophilus* [7]. The *als* gene encodes acetolactate synthase enzyme (ALS) containing 560 amino acid residues. The PFLA, pyruvate-formate lyase activating enzyme (267 a.a.), and ADHB, alcohol dehydrogenase enzyme (345 a.a.), were encoded by *pflA* and *adhB* genes, respectively. The genes containing ribosome binding sites were amplified with appropriate primers (see Fig. 2a–c), ligated into pJET1.2 vector, and transformed into *E. coli* MC1022.

Construction of the *als* Overexpression Vector, p131

The pJET1.2 plasmid (p121) containing the *als* fragment was digested with restriction enzymes (Fig. 3a), and the *als* DNA fragment purified from the agarose gel. The plasmid pNZ276 was also digested (Fig. 3b), and the approximate 4261 bps fragment was purified from the gel. The *AccIII*/*XhoI*-digested *als* fragment was ligated into *AccIII* and *XhoI* sites of pNZ276, and transformed into *E. coli* MC1022. Colonies were screened by PCR using *AlsF3* and *AlsR2* primers, and then confirmed with *AccIII* and *XhoI* digestion. The 1743 bp fragment containing the *als* gene cloned into pNZ276 vector under control of *lacA* promoter. After the confirmation with the DNA sequencing, the resulting plasmid was named as p131 (Fig. 4; Table 1). *E. coli* strain harboring this plasmid was designated as E-MMG131 (Table 1).

Construction of the *pflA* Overexpression Vector, p132

The 801-bp length *pflA* fragment was isolated by digestion with *AccIII* and *XhoI* from transformant pJET1.2 plasmid (p122; Table 1) and ligated into *AccIII*- and *XhoI*-digested pNZ276 plasmid and transformed into *E. coli* MC1022. Following colony screening by PCR with *PflAF* and *PflAR* primers and sequencing analysis, the plasmid containing *pflA* fragment was named as p132 (Fig. 4; Table 1). *E. coli* strain harboring this plasmid was designated as E-MMG132 (Table 1).

Fig. 2 PCR amplification of fragments containing **a** *als*, **b** *pflA*, and **c** *adhB* genes. Lane *M* DNA marker; lanes 1 and 2 are PCR product amplified with the appropriate primers

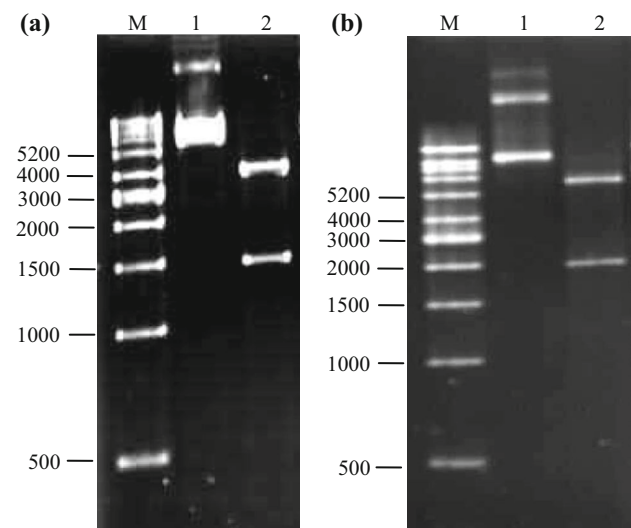
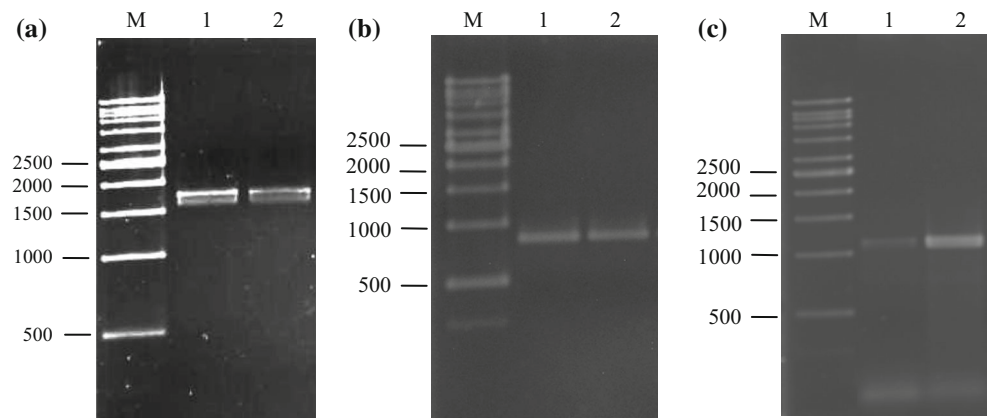


Fig. 3 **a**. Agarose gel electrophoresis of *AccIII*- and *XhoI*-digested pJET1.2 vector (p121) containing *als* fragment, Lane 1 is uncut plasmid DNA; Lanes 2 is the plasmid p121 digested with *AccIII* and *XhoI* **b** Agarose gel electrophoresis of the digestion of pNZ276 plasmids with *AccIII* and *XhoI*. Lane 1 is uncut pNZ276 plasmid DNA; Lane 2 is the plasmid pNZ276 digested with *AccIII* and *XhoI*. Lane *M* DNA marker

Construction of the *adhB* Overexpression Vector, p134

The 1078-bp length PCR amplified *adhB* fragment (Fig. 2c) was ligated into pJET1.2 plasmid forming p124 (Table 1). The *adhB* gene was then excised with *PstI* and *XhoI* restriction enzymes from transformant pJET1.2 plasmid. Purified fragment from agarose gel was cloned into *PstI*- and *XhoI*-digested pNZ276 plasmid and transformed into *E. coli* MC1022. After PCR, restriction and sequence analyses, the plasmid containing *adhB* fragment was named as p134. *E. coli* strain harboring this plasmid was designated as E-MMG134 (Table 1).

The overexpression plasmids p131, p132, and p134 (Fig. 4) were electroporated separately into *S. thermophilus*

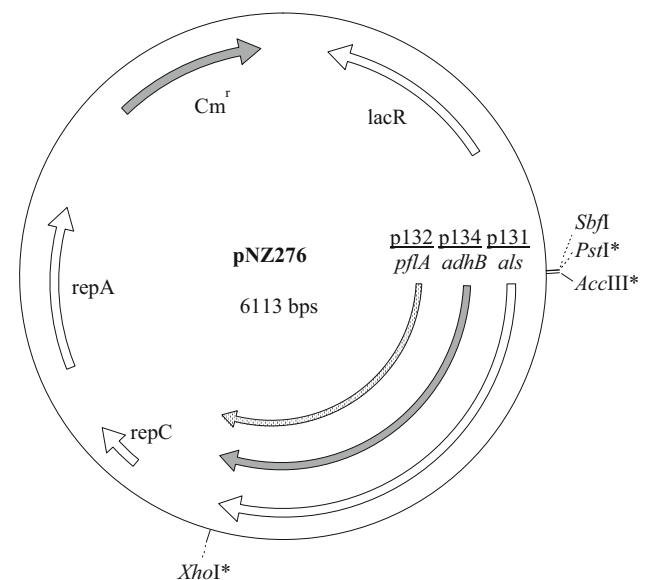


Fig. 4 Schematic representation of plasmids p131, p132, and p134. *Restriction endonucleases were used to construct

NCFB 2393 strain, and resulting strains named as S-MMG131, S-MMG132, and S-MMG134, respectively (Table 1).

Analysis of Metabolites Production

Metabolic engineering strategies aimed to enhance desired metabolite production by the over production of relevant enzymes. The end of the molecular construction, *als*⁺⁺, *pflA*⁺⁺, and *adhB*⁺⁺ overexpressed *S. thermophilus* strains were generated and their end product diversion behaviors were analyzed. In order to evaluate the desired metabolite production, the small-scale cell cultures (10 mL) of the reference and the constructed strains carrying the overexpression vector were grown with the lactose substrate. The levels of end products produced from NCFB 2393 strain were used as a reference. Depending on the overexpressed

gene, the conversion of the end metabolites may be redirected (Table 2). The supernatants after overnight growth were analyzed by HPLC to measure the effect of the overexpression of the *als*, *pflA*, or *adhB* genes on the metabolic activity. The overexpression of the *als* gene increases the level of ALS enzyme and this enzyme is regulated by the increasing amount of α -acetolactate, diacetyl, and 2,3-butanediol. The result of metabolite screening showed that the highest amount of end product was produced through 2,3-butanediol conversion. The amount of acetoin, the intermediate metabolite produced from α -acetolactate and diacetyl, slightly increased with the overexpression of the *als* gene (Table 2).

The overexpression of *pflA* gene resulted in increased amount of pyruvate diversion to formate. The pathway between pyruvate and formate directed to acetyl-coenzyme, and pyruvate is not the only route to convert formate. As expected, the overexpression of *pflA* gene increased formate accumulation slightly, and *pflA*⁺⁺ strain showed similarities with the reference strain (Table 2).

Reduction of acetaldehyde to ethanol terminates the fermentative pathways of various ethanol producing microorganisms. This reaction was catalyzed by NAD(P)H-dependent alcohol dehydrogenases enzyme (see Fig. 1). There genes (*adhA*, *adhE*, and *adhB*) were described in acetaldehyde to ethanol pathway. The *adhA* and *adhE* genes are pseudogenes and *adhB* gene overexpressed in *adhB*⁺⁺ strain. When compared to the reference strain, the ethanol production was increased 5.4-fold but acetaldehyde, formate, pyruvate, and lactate production were decreased with the overexpression of the *adhB* gene (Table 2).

Discussion

Streptococcus thermophilus is a thermophilic species of lactic acid bacteria that can be used as a dairy starter culture for the production of the yoghurt with the combination

of *Lactobacillus delbrueckii* subsp. *bulgaricus* and a variety of cheese. Enormous gene inactivation and the loss of the most virulence determinants support the ‘generally recognized as safe’ status of *S. thermophilus* [7]. The aroma and the flavor of yoghurt are fundamentally related to the production of non-volatile and volatile acids and carbonyl compounds [6]. Yoghurt starter cultures generate lactate, acetaldehyde, diacetyl, ethanol, propanone, and 2-butanone metabolites via the pyruvate pathway. Acetaldehyde is responsible for a typical yoghurt aroma and it is the most prominent compound with the concentrations in the range of 5–21 mg/L in the final product [25, 26]. The metabolite formation abilities of starter cultures are important to determine the properties of the fermented product. To improve the metabolite formation abilities of starter cultures by redirecting the metabolic pathways, metabolic engineering strategies are widely used. Redirection of metabolic pathways is important for industrial compound production.

In this study, we have overexpressed the *als*, *pflA*, and *adhB* genes individually in *S. thermophilus* NCFB 2393. The metabolites were analyzed by HPLC from the cultures grown in LM17 broth medium. The improving level of 2,3-butanediol was obtained by overexpressing the *als* gene. The level of formate increased slightly by overexpressing the *pflA* gene. The overexpression of the *adhB* gene resulted in remarkable increase in the ethanol level. The overexpression of *adhB* gene in wild-type strain made a clear metabolic shift in the production of metabolic end product.

Completed genome sequence of *S. thermophilus* showed that *butA* gene (Fig. 1) taking role in acetoin and 2,3-butanediol productions [16]. However, our results showed that the overexpression of the *als* gene increased 2,3-butanediol production in S-MMG131 strain. This strain is also able to produce detectable amount of acetoin compared to other strains (Table 2). Therefore, the *als* gene overexpression with the overexpression of the *aldB* gene

Table 2 Metabolite production of *S. thermophilus* strains in LM17 medium and analysis by HPLC

Strain	Produced end metabolites (μ M)						
	Formate ^a	Pyruvate ^a	Lactate ^a	Acetoin ^a	Acet-aldehyde ^b	Ethanol ^c	2,3-Butanediol ^c
NCFB2393	5.49 \pm 0.62	1.46 \pm 0.02	0.97 \pm 0.02	ND	28.79 \pm 2.23	6.38 \pm 1.04	ND
S-MMG131 (<i>als</i>)	0.11 \pm 0.01	0.016 \pm 0.01	0.03 \pm 0.01	1.41 \pm 0.04	3.49 \pm 0.19	ND	33.315 \pm 0.55
S-MMG132 (<i>pflA</i>)	6.76 \pm 0.96	0.41 \pm 0.12	ND	ND	29.54 \pm 2.75	3.76 \pm 0.53	ND
S-MMG134 (<i>adhB</i>)	3.91 \pm 0.64	0.23 \pm 0.01	ND	ND	3.097 \pm 1.01	35.08 \pm 4.82	ND

ND not detected

^a Formate, pyruvate, lactate, and acetoin were detected at 210 nm

^b Acetaldehyde was detected at 277 nm

^c Ethanol and 2,3-butanediol were detected at 190 nm

could increase 2,3-butanediol production. In the other study, the overexpression of the *als* ve *ar* (acetoin reductase) genes in *Klebsiella pneumonia* resulted in the high level of 2,3-butanediol production for industrial use [15]. α -ALS, α -acetolactate decarboxylase (ALDC), and acetoin reductase (AR) enzymes catalyze the production of acetolactate from pyruvate, acetoin from acetolactate, and 2,3-butanediol from acetoin, respectively.

In *S. thermophilus*, threonine is converted into acetaldehyde and glycine by threonine aldolase activity [10]. The serine hydroxymethyltransferase enzyme, which has threonine aldolase activity, was encoded by the *glyA* gene and the inactivation of the *glyA* gene resulted in complete loss in acetaldehyde formation during fermentation. When the growth medium was supplemented with L-threonine, acetaldehyde production increased [10]. We previously observed an increase in acetaldehyde production when *glyA* gene was overexpressed (Akyol et al. unpublished result). In *Zymomonas mobilis*, the pyruvate decarboxylase (Pdc) enzyme catalyzes the conversion of pyruvate to acetaldehyde (see Fig. 1), which is finally reduced to ethanol by alcohol dehydrogenase [37]. The NADH oxidase (Nox) is known to decrease lactate production by NADH-dependent Ldh under aerobic conditions [12], leading to increased pyruvate availability for alternative reactions. Both *pdc* and *nox* genes were overexpressed in *L. lactis* and Pdc overproduction rerouted the pyruvate metabolism towards acetaldehyde and ethanol. In the reference *L. lactis* strain, lactate and acetate were main fermentation products under aerobic condition while a clear decrease of lactate production and increased acetoin production were resulted in *nox* overexpressed strains [8].

The pyruvate-formate lyase (Pfl) enzyme catalyzes the non-oxidative transformation of pyruvate into acetyl-coenzyme A and formate. The regulation of the *pfl* expression and Pfl activity has been intensively studied in *S. bovis* [4, 5] and *L. lactis* [3, 22]. Under anaerobic conditions and in the presence of slowly fermentable sugars, Pfl is responsible for the shift from homolactic to mixed-acid fermentation in *L. lactis*. Formate production was not detected, which suggests that Pfl is irreversibly inactivated by the exposure to oxygen during preparation of the incubation mixtures [3, 17, 23]. In *S. bovis*, it was shown that the *pfl* expression is also dependent on the nature of the fermentable carbon source via a CcpA-dependent mechanism [5]. In the strain *S. thermophilus* LMG18311, a catabolite-responsive element (cre box) was detected the upstream of the *pfl* (sequence TGTAAGCGGTTACT, at –92 bp, the upstream of the putative ATG start codon), suggesting that the nature of the carbon source would also modulate the *pfl* expression in *S. thermophilus*. Proteomics and transcriptional studies of the *pfl* gene were made and the Pfl enzyme was just at the detectable level in M17

grown *S. thermophilus*. When *S. thermophilus* cultures grown exponentially in LM17 medium were shifted to milk medium for 15 min, a strong increase in the abundance of the *pfl* mRNA was observed [13]. The *pfl* transcript abundance was maximal during the exponential phase but drastically decreased at the start of stationary phase. The proteome analysis showed that the same trend was observed at the protein level so Pfl was the strongest overexpressed protein of *S. thermophilus* LMG18311 during exponential growth in milk [13]. In a mixed-culture transcriptome analysis of *S. thermophilus* and *L. bulgaricus*, it was found that the *pfl* ve *pflA* were higher expressed in mixed culture, especially in the first exponential phase (3.0- and 4.1-fold, respectively) compared to monocultures. In the pure *S. thermophilus* culture, the gene *pflA* and the pathway for purine were upregulated in the transition phase compared to the first exponential phase [30]. *S. thermophilus* provide *L. bulgaricus* with crucial components for purine nucleotide biosynthesis, including the precursor formic acid and the cofactor folic acid.

In conclusion, we have successfully cloned the *als*, *pflA*, and *adhB* genes of a *S. thermophilus* strain. The high level of the enzymes increased the production of 2,3-butanediol and ethanol metabolites. These metabolites can be purified for the industrial use. Additionally, some wild *S. thermophilus* strains [14] can be transformed with these created constructs in order to see the effects of overexpression on metabolite formation.

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