

Consolidated Bioprocessing for Bioethanol Production by Metabolically Engineered *Bacillus Subtilis* Strains

Fatemeh Maleki

NIGEB: National Institute for Genetic Engineering and Biotechnology

Mohammad Changizian

NIGEB: National Institute for Genetic Engineering and Biotechnology

Narges Zolfaghari

NIGEB: National Institute for Genetic Engineering and Biotechnology

Sarah Rajaei

NIGEB: National Institute for Genetic Engineering and Biotechnology

Kambiz Akbari Noghabi

NIGEB: National Institute for Genetic Engineering and Biotechnology

Hossein Shahbani Zahiri (✉ shahbani@nigeb.ac.ir)

NIGEB <https://orcid.org/0000-0002-7417-0307>

Research

Keywords: Bacillus subtilis, Bioethanol, Consolidated bioprocessing, Metabolic engineering

Posted Date: March 11th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-297375/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Background: Bioethanol produced by fermentative microorganisms is regarded as an alternative to fossil fuel. Bioethanol to be used as a viable energy source must be produced cost-effectively by removing expense-intensive steps such as the enzymatic hydrolysis of substrate. Consolidated bioprocessing (CBP) is believed to be a practical solution combining saccharification and fermentation in a single step catalyzed by a microorganism. *Bacillus subtilis* with innate ability to grow on a diversity of carbohydrates seems promising for affordable CBP bioethanol production using renewable plant biomass and wastes.

Results: In this study, the genes encoding alcohol dehydrogenase from *Z. mobilis* (*adh_Z*) and *S. cerevisiae* (*adh_S*) were each used with *Z. mobilis* pyruvate decarboxylase gene (*pd_{CZ}*) to create ethanologenic operons in a lactate-deficient (Δ *ldh*) *B. subtilis* resulting in NZ and NZS strains, respectively. The *S. cerevisiae* *adh_S* caused significantly more ethanol production by NZS and therefore was used to make two other operons including one with double copies of both *pd_{CZ}* and *adh_S* and the other with a single *pd_{CZ}* but double *adh_S* genes expressed in N(ZS)2 and NZS2 strains, respectively. In addition, two fusion genes were constructed with *pd_{CZ}* and *adh_S* in alternate orientations and used for ethanol production by the harboring strains namely NZ:S and NS:Z, respectively. While the increase of gene dosage was not associated with elevated carbon flow for ethanol production, the fusion gene *adh_S:pd_{CZ}* resulted in more than two times increase of productivity by strain NS:Z as compared with NZS during 48 h fermentation. The CBP ethanol production by NZS and NS:Z using potatoes resulted in 16.3 g/L and 21.5 g/L ethanol during 96 h fermentation, respectively.

Conclusion: In this study for the first time, *Bacillus subtilis* was successfully used for CBP ethanol production with *S. cerevisiae* alcohol dehydrogenase. The results of the study provide insights on the potentials of *B. subtilis* for affordable bioethanol production from inexpensive plant biomass and wastes. However, the potentials need to be improved by metabolic and process engineering for higher yields of ethanol production and plant biomass utilization.

Background

There has been a growing interest in using agricultural wastes and by-products as abundant, non-expensive, and non-food feedstocks for bioethanol production. Plant biomass is mainly composed of cellulose and starch that need to be hydrolyzed into fermentable sugars before being used for ethanol production [1]. Natural ethanologenic microorganisms like *Saccharomyces cerevisiae* and *Zymomonas mobilis* can utilize only a few simple sugars but, of course, not starch and other polysaccharides. Therefore, the polymeric plant biomass needs to be converted into consumable sugars for fermentation by these microorganisms. In order to reduce the costs, it is desirable to obtain an ethanologenic strain that can consume polymeric carbohydrates on its own to integrate the saccharification and fermentation in a consolidated process. For this purpose, several attempts have been made to transform ethanologenic strains by the expression of exogenous enzymes and pathways to broaden the range of substrates they can utilize for ethanol production [2–4]. However, there are still drawbacks as to the efficiency of

ethanologenic microorganisms for the production and secretion of exogenous enzymes as well as their tolerance against the toxicity and inhibitory effects of biomass hydrolysates. On the other hand, it is quite tempting to convert non-ethanologenic microorganisms that are able to grow on a wide range of carbon sources into ethanol producers [5, 6]. In this regard, gram-positive bacteria seem to be appropriate candidates due to having beneficial properties such as the ability to survive at high temperature and low pH conditions, and tolerance of high concentrations of sugar, salt, and ethanol. *Bacillus subtilis* is a gram-positive bacterium that is generally recognized as safe (GRAS) and is amenable to genetic manipulation as well [7]. This bacterium with an optimum temperature of 37°C can grow at temperatures up to 50°C and ferments many carbohydrates from mono-, di-, oligo-, and polysaccharides. In this regard, the ability of *B. subtilis* to utilize starch, xylan, galactan, pullulan, arabinan, rhamnogalacturonan, and pectin is quite interesting when it comes to converting plant biomass wastes into biotechnological products with added value. The capability of *B. subtilis* to survive on different carbohydrates is largely due to the production and efficient secretion of various hydrolytic enzymes by this bacterium. Therefore, it is quite intriguing to take advantage of the ability to extend the substrate range of *B. subtilis* by the expression of exogenous genes encoding novel enzymes required for complete degradation of plant biomass [8]. However, in order for bioethanol production, *B. subtilis* needs to be improved by genetic manipulation and pathway engineering as the inherent capacity of this bacterium for ethanol production is quite negligible. The only successful attempt to develop an ethanologenic strain of *B. subtilis* has been reported by Romero et al. [6]. They managed to create the strain by engineering an exogenous ethanol pathway using heterologous expression of *Z. mobilis* genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*). In addition, they had to knock out the native genes encoding lactate dehydrogenase (*ldh*) and acetolactate synthase (*alsS*) to obstruct lactate and 2,3- butanediol production, respectively, as major fermentation products of *B. subtilis*. To this end, the *ldh* was disrupted by the insertion of *Z. mobilis pdc* and *adhB* genes; and *alsS* was knocked out by the insertion of *E. coli udhA* encoding a transhydrogenase to balance NADH/NADPH ratio. By the redirection of the fermentative metabolism, the resulting strain was able to produce 8.9 g/L ethanol from 20 g/L glucose during 9 days of fermentation under nonaerated conditions. Despite the interesting results, the ethanol titer needs to be improved significantly to become economically viable. In addition, the positive point of *B. subtilis* compared to natural ethanologenic strains such as *S. cerevisiae* is that the bacterium can utilize polysaccharides, and thus it might be used in CBP systems for affordable bioethanol production from plant biomass without the need to enzymatic pretreatments. With regard to this point, the current study was conducted to evaluate the potential of *B. subtilis* for bioethanol production from untreated potatoes in a consolidated bioprocess setting (Fig. 1). For this purpose, lactate production as a significant rival pathway had to be eliminated. The ethanologenic *B. subtilis* strains were engineered using the genes encoding *Z. mobilis* pyruvate decarboxylase (*pdc*) and the alcohol dehydrogenase of *Z. mobilis* (*adhB*) and *S. cerevisiae* (*adhI*). Also, the influence of additional copies of *pdc* and *adh*, as well as their fusions encoding bifunctional enzymes were analyzed on bioethanol production by *B. subtilis*. The resulting strains were investigated for ethanol production on glucose as well as for CBP ethanol production on potatoes.

Results

Inactivation of lactate dehydrogenase

The gene encoding lactate dehydrogenase (*ldh*) was disrupted by chromosomal insertion of pDHT*ldh* using homologous recombination. *B. subtilis* WB600 was transformed with pDHT*ldh* and the resulting transformant, named *B. subtilis* WBN, was selected on an LB plate with chloramphenicol. The authenticity of homologous recombination and disruption of *ldh* in WBN strain was confirmed by PCR using the *dldh*-F and *dldh*-R primers. The *dldh*-F was designed to anneal to WBN chromosomal DNA just upstream to *ldh* sequence while the reverse primer *dldh*-R was designed according to the *lacI* gene of pDHT*ldh*. The PCR product of about 800 bp was sequenced, revealing that it contained partial sequences from both WB600 *ldh* and the *lacI* of pDHT*ldh*. The result confirmed that pDHT*ldh* was correctly inserted in WBN chromosome by a Campbell-like mechanism, resulting in the disruption of the *ldh* gene [9, 10].

Comparison of *S. cerevisiae* *adhI* and *Z. mobilis* *adhB*

The efficiency of *S. cerevisiae* *adhI* for ethanol production was analyzed against *Z. mobilis* *adhB* using NZ and NZS strains. The strains were cultured in 2YT medium containing 4 % glucose under limited aeration conditions. The strain NC harboring pHY300PLK was used as a control. The results showed that NZ produced 3.7 g/L ethanol but NZS was more efficient producing 4.7 g/L ethanol during 48 hours of incubation. The ethanol production of NC during the same time was about 0.1 g/L (Fig. 2a). However, during the next 48 hours of fermentation, ethanol concentration in the culture mediums of NZ and NZS did not change significantly but that of NC was increased to about 0.6 g/L (Fig. 2b). Although the ethanol production by normal *B. subtilis* is quite negligible, in the NC strain due to the lack of lactate production, the carbon flux could partly be allotted to ethanol production. The growth of NZ and NZS did not seem to be adversely influenced by the synthetic ethanol pathways. Both strains efficiently utilized more than 90 % of initial glucose during 48 h of fermentation. In contrast, NC just consumed less than 50 % of the added glucose. Given the comparable growth of the strains, the higher glucose consumption of NZ and NZS can be attributed to the function of the exogenous ethanol pathways (Fig. 2). The results showed that *adh_S* was remarkably more efficient than *adh_Z* resulting in 30 % more ethanol production by *B. subtilis*.

Ethanol production by NZS under various aeration conditions

The effect of aeration on the growth and ethanol production was studied under normal, limited, and biphasic aeration conditions using the strain NZS with 60 g/L glucose. Interestingly, *B. subtilis* NZS was able to produce ethanol even under the high oxygen transfer rate of normal aeration conditions (Fig. 3). However, the ethanol accumulation under such conditions was less than half of those obtained under limited and biphasic aeration. The results showed that under high aeration, much of the added glucose was consumed for growth (Fig. 3). The ethanol concentration at the end of fermentation was 7.7 g/L and the ethanol production yield from the consumed glucose was merely about 29 % of the theoretical maximum. Under the limited aeration conditions, the growth was lowered providing the ethanol pathway with the chance to channel more of the carbon flow into ethanol production. The ethanol concentration was about 11 g/L after 96 h and the ethanol production yield was improved to about 45 %. When cultures were conducted under the biphasic aeration conditions, the overall glucose consumption was higher than other

aeration conditions, and the ethanol production was slightly improved to about 11.8 g/L. However, the ethanol production yield was about 42 % of the theoretical maximum indicating that the synthetic ethanol pathway at the existing expression level was not so efficient as to appropriate a larger portion of the carbon metabolism.

Combinatorial effects of aeration, temperature, and shaking on ethanol production

The ethanol production by NZS was evaluated in cultures with 50 g/L glucose under limited and normal aeration conditions at two temperatures of 30 and 37 °C and two shaking rates of 120 and 180 rpm (Fig. 4). The results showed that at 30 °C and 120 rpm, there was no significant difference in ethanol production with either limited or normal aeration resulting in about 11 g/L ethanol accumulation after 96 h (Fig. 4a). At the same temperature of 30 °C but elevated shaking rate of 180 rpm, growth under the limited aeration conditions resulted in a significantly higher ethanol accumulation of 11 g/L against 8.9 g/L ethanol of the normal aeration (Fig. 4b). The ethanol accumulation at 37 °C was more significantly affected by shaking rate and aeration conditions so that at 120 rpm under normal aeration conditions, a high ethanol concentration of 10 g/L was accumulated just during 48 h of incubation while only 2.3 g/L of ethanol was produced with limited aeration during the same incubation time (Fig. 4c). Finally, the ethanol production by NZS was studied at 37 °C and 180 rpm. The results showed that the culture conditions were more favorable for ethanol production under limited aeration resulting in ethanol production of 12.3 g/L. However, even with normal aeration, a rather high amount of ethanol (8.3 g/L) could be produced by the strain (Fig. 4d). The results presented in figure 4 indicate that aeration, shaking and temperature exert a combinatorial effect on the ethanol production of NZS. These parameters manage the yield and productivity of ethanol production by influencing the growth rate and metabolism of strain NZS as well as the activity of ethanologenic enzymes. As such, the highest ethanol productivity (0.21 g/L/h) was achieved under the normal aeration at 37 °C and 120 rpm but the highest yield (48 % of the theoretical maximum) was obtained under limited aeration at 37 °C and 180 rpm. The figures for yield and productivity seem quite remarkable, given that just a small inoculum was used for culture mediums and the cells had to produce ethanol while growing on a total glucose concentration of 50 g/L.

CBP Ethanol production by NZS using untreated potatoes as a substrate

With respect to the ability of *B. subtilis* to produce extracellular hydrolases, strain NZS was evaluated for CBP ethanol production using untreated potatoes as a typical starchy substrate. The results showed that NZS could grow on all tested concentrations of DGP using its native hydrolysis capacity and produced ethanol by CBP (Fig. 5). After 96 h of fermentation, the concentration of ethanol in the culture mediums with 50, 100, and 150 g/L DGP was 9.6 g/L, 12.7 g/L, and 16.3 g/L while about 26 g, 45 g, and 70 g of the initial DGP, respectively, was solubilized (data not shown). The yield of ethanol production using 50, 100, and 150 g/L DGP was about 65 %, 50 %, and 41 % of the theoretical maximum, estimated roughly based on the solubilized biomass.

Effects of gene copy number and fusions on ethanol production

The effects of the copy number and relative activity of *pd_Z* and *adh_S* on ethanol production were analyzed using ethanologenic plasmids either containing more than one copy of the genes or having a gene fusion instead of an operon. The strains N(ZS)2, NZS2, NS:Z, NZ:S as well as the control strain NC were cultured in 2YT medium with 60 g/L glucose. The ethanol concentration, growth, and residual glucose were determined at 24 h intervals (Fig. 6). The highest ethanol concentration of 9.6 g/L was detected just after 48 h in the culture medium of strain NS:Z with a productivity of 0.2 g/L/h and a yield of 31 %. The next ethanol producer with 8.7 g/L during 96 h of incubation was NZS resulting in a productivity of 0.09 g/L/h and a yield of 28 %. Although the maximum ethanol production of strain NZS2 was 5.66 g/L, the strain with a productivity of 0.12 g/L/h was revealed to be faster than NZS in ethanol production and glucose consumption during the first 48 h of incubation. Strains NS:Z, NZS2, and NZS consumed about 100 %, 92 %, and 87 % of the initial glucose (60 g/L) during 48 h of incubation of which one-third (19 g), one-fifth (11.2 g), and one-sixth (8 g) were converted to ethanol, respectively. As for NS:Z and NZS2, the growth peak was temporally corresponding with ethanol production peak and glucose depletion. Therefore, it may be assumed that the relative activity of pyruvate decarboxylase and alcohol dehydrogenase in the strains were well suited to the metabolism of the host resulting in concurrent growth and ethanol production. In contrast, strains NZ:S and N(ZS)2 were adversely affected by the expression of ethanologenic enzymes. As for strain NZ:S, in particular, the growth, ethanol production, and glucose consumption were severely inhibited as a result of the expression of fusion *pd_Z:adh_S*. From the results it could finally be inferred that strain NS:Z was more efficient than other strains in ethanol production, and the lack of enough glucose might have been a major limiting factor for growth and more ethanol production of the strain during the late 48 hours of fermentation.

CBP ethanol production by strain NS:Z using untreated potatoes

Given the favorable characteristics of NS:Z as an ethanologenic strain, it was evaluated for CBP ethanol production on the untreated potatoes. For this purpose, cultures were conducted in 2YT medium containing 100 g/L and 150 g/L DGP. The results showed that NS:Z was able to grow in such highly viscous mediums, and surprisingly produced high concentrations of ethanol by CBP (Fig. 7). The ethanol production in the mediums with 100 g/L and 150 g/L DGP was not significantly different by 48 hours of fermentation, regardless of the initial DGP concentration. However, at the end of fermentation (96 h), the ethanol concentration increased to 12.5 g/L and 21.4 g/L in cultures with 100 g/L and 150 g/L DGP, respectively. In the other words, 73 percent more ethanol was accumulated in the culture with the higher initial DGP concentration. The determination of residual solids in the culture mediums indicated that about 78 g/L and 69 g/L out of the 100 g/L and 150 g/L initial DGP, respectively, have been solubilized over the time course of fermentation. Therefore, the ethanol production yield was estimated at 28 % and 54 % of the theoretical maximum with 100 g/L and 150 g/L initial DGP, respectively. The impact of strain NS:Z on the solubilization of DGP and the fluidity of the culture medium was inspected by viscosity analysis. While the viscosity of the uninoculated culture medium with 150 g/L DGP remained almost unchanged at 86.25 (P), the viscosity of the culture medium inoculated with NS:Z was significantly decreased to 1.65 (P) during the 96 h fermentation (data not shown). The

results showed that NS:Z in a tiny inoculation (initial OD_{600 nm} of 0.1) was able to propagate in the highly viscous medium containing 150 g/L DGP, reducing the viscosity by 52 times as a result of its metabolism and secretion of hydrolytic enzymes.

Discussion

B. subtilis can survive on a range of different substrates due to its metabolic diversity and robust systems for the production and secretion of enzymes [11–14]. The bacterium exhibits low nutrient requirements and remarkable tolerance to high concentrations of salt and solvents. These features are of significant importance where bioconversion of low-cost feedstocks to value-added biocommodities is aimed [15, 16]. While *S. cerevisiae* and *Z. mobilis*, as the most important ethanologenic organisms, are only able to consume few carbohydrates, *B. subtilis* can utilize a diversity of carbon sources. The ability to consume polysaccharides makes *B. subtilis* a promising candidate for the development of ethanologenic strains for bioconversion of plant biomass wastes. In this respect, *B. subtilis* has an elaborate system for the consumption of starch biomass, through which the starch is hydrolyzed by extracellular amylolytic enzymes to release maltose and maltodextrins. Maltose is taken up by the phosphotransferase system (PTS) and hydrolyzed into glucose and glucose-6-P by the cytoplasmic phospho- α -1,4-glucosidase (MalA). Maltodextrins are taken up by a specific ABC transporter without phosphorylation and are degraded into glucose by cooperative actions of a cytoplasmic maltogenic amylase (YvdF), a maltose phosphorylase (YvdK), and a glucosidase (MalL) [17]. Therefore, the use of an ethanologenic *B. subtilis* in CBP system would eliminate the need for gelatinization, liquefaction, and saccharification steps that are currently required for ethanol production from starchy biomass by *S. cerevisiae* (Fig. 1).

The only report of successful development of an ethanologenic *B. subtilis* strain has been published by Romero et al. [6]. They used *Z. mobilis pdc* and *adhB* genes to make a synthetic ethanologenic operon in *B. subtilis*. The operon was inserted into the *B. subtilis* chromosomal *ldh* encoding lactate dehydrogenase, which is responsible for reducing pyruvate to lactate. Consequently, the lactate production as the main fermentation product of *B. subtilis* was disrupted, and instead, the ethanol operon could be expressed under the control of the *ldh* promoter. However, the growth and glucose consumption of the resulting strains were significantly decreased by 70 and 65 %, respectively. In this regard, the researchers managed to find out that *B. subtilis* lactate dehydrogenase was able to utilize both NADH and NADPH as cofactor, balancing the cellular concentration of their reduced form. In the ethanologenic Δ *ldh* strain, while the NADH-oxidation activity of lactate dehydrogenase could be fulfilled by *Z. mobilis adhB*, the NADPH oxidation remained unattended, resulting in an unbalanced NADP⁺/NADPH ratio and consequently a lowered growth rate. To tackle the problem as well as to block butanediol production as a significant rival pathway for ethanol production by *B. subtilis*, the gene coding for *E. coli* transhydrogenase (*udhA*) was inserted by the researchers into the acetolactate synthase gene (*als*) [18]. The transhydrogenase mediates the reciprocal transfer of hydride between NAD(H) and NADP(H), restoring the NADP⁺/NADPH ratio in the absence of lactate dehydrogenase activity. The resulting strain BS37 showed 22% and 59% improvements in the growth rate and glucose consumption, respectively. Consequently, the ethanol production by the strain was raised to 8.9 g/L in a culture with 20 g/L glucose during 9 days of incubation under nonaerated conditions

in minifleakers at 35°C, 100 rpm, and pH 7 [6]. Apart from the study conducted by Romero et al., other attempts for making ethanologenic strains from gram-positive bacteria have not been as successful [5, 19–23].

Lactate is the major fermentation product of *B. subtilis* and is produced at substantially higher amounts than acetate and 2,3-butanediol as the next most abundant fermentation products of the bacterium [24]. As in Romero et al. study, the deactivation of the lactate pathway proved in the present study to be crucial for ethanol production by *B. subtilis*, as neither the intrinsic ethanol pathway nor the engineered heterologous pathway was able to elicit noticeable ethanol production in the parental WB600 strain. The resulting Δ/dh strain (WBN) was successfully used for ethanol production using a synthetic operon containing *S. cerevisiae* alcohol dehydrogenase (*adh*), and *Z. mobilis* pyruvate decarboxylase (*pdc*). The strain (NZS) was able to grow and produce ethanol under various aeration conditions. It is noticeable that the problems associated with ethanol production in the Romero et al. study might have been related to the nonaerated culture conditions. In the present study, cultures were conducted under aerated conditions obviating the need for an exogenous transhydrogenase to deal with the unbalance of redox equivalents that may occur with non-aerated cultures in the absence of lactate dehydrogenase activity. The growth of the engineered strains under aerated conditions resulted in high concentrations of ethanol in a shorter period of fermentation. Under such conditions, the ethanologenic strains grew well on potatoes and produced ethanol in CBP at higher concentrations than those obtained with glucose. In this study, the improvement of growth and ethanol production by fusing *Z. mobilis* pyruvate decarboxylase (PDC) to *S. cerevisiae* alcohol dehydrogenase I (ADH) in the ADH- PDC configuration may have resulted from a favorable change in the relative activity of the enzymes. It has been shown that the development of enzyme fusions is an effective approach for making bi- or multi-functional enzymes with improved desirable characteristics [25, 26]. The catalytic activity of enzymes is highly likely to be altered by fusions so that it may be increased or decreased for both enzymes or increased for one but decreased for the other enzyme. In addition, the orientation of domains in a fusion enzyme may significantly affect the enzyme properties [27, 28].

Conclusions

B. subtilis exhibits important features to be used for bioethanol production from biomass. In this study, several ethanologenic strains of *B. subtilis* were developed and evaluated for aerobic bioethanol production. The results obtained in this study suggest that *B. subtilis* shows potentials to be developed into a significant producer of CBP ethanol from low-cost agricultural wastes. In this context, the capability of *B. subtilis* to utilize polymeric carbohydrates may be extended by the expression of synthetic genes encoding select enzymes required for efficient digestion of complex plant biomass.

Methods

Strains

The strains, plasmids, and primers used in this study are listed in Table 1. The strains of *Zymomonas mobilis* 1718 and *Saccharomyces cerevisiae* 5080 were purchased from PTCC (Tehran, Iran). *Z. mobilis*

was cultured in a medium composed of 1 % Bacto peptone, 1 % yeast extract, 2 % glucose at 30°C. *S. cerevisiae* was grown at 26°C in a culture medium with 1 % glucose, 0.5 % peptone, 0.3 % yeast extract, 0.3 % malt extract. *Escherichia coli* DH5α and *B. subtilis* WB600 were available from the laboratory stock and routinely cultured on LB (Luria-Bertani) medium (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl) at 37°C. *E. coli* DH5α was used as a host for the construction of recombinant plasmids containing ethanologenic operons. The transformation of *E. coli* DH5α was performed by the heat shock method and the transformants were selected on LB agar plates with ampicillin according to standard protocols [29]. *B. subtilis* WB600 was used for the creation of ethanologenic strains by deletion of *ldh* (encoding lactate dehydrogenase) and transformation of the resulting strain (*B. subtilis* WBN) with recombinant plasmids using natural transformation according to Anagnostopoulos and Spizizen [30].

Table 1
list of strains, plasmids, and primers used in this study

Strains	Description	Source
<i>E. coli</i> DH5α	F ⁻ φ80 <i>dlacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ , m ⁺) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	Invitrogen
<i>Z. mobilis</i>	Ethanologenic strain	PTCC 1718
<i>S. cerevisiae</i>	Ethanologenic strain	PTCC 5080
<i>B. subtilis</i> WB600	<i>trpC2 nprE aprE epr bpr mpr nprB</i> ; Em ^r	[31]
<i>B. subtilis</i> WBN	<i>Bacillus subtilis</i> WB600, <i>ldh::pDHtldh</i>	This study
<i>B. subtilis</i> NC	WBN harboring pHY300PLK	This study
<i>B. subtilis</i> NZ	WBN harboring pHY <i>pd_{CZ}adh_Z</i>	This study
<i>B. subtilis</i> NZS	WBN harboring pHY <i>pd_{CZ}adh_S</i>	This study
<i>B. subtilis</i> NZS2	WBN harboring pHY <i>pd_{CZ}(adh_S)₂</i>	This study
<i>B. subtilis</i> N(ZS) ₂	WBN harboring pHY(<i>pd_{CZ}adh_S</i>) ₂	This study
<i>B. subtilis</i> NZ:S	WBN harboring pHY <i>pd_{CZ}:adh_S</i>	This study
<i>B. subtilis</i> NS:Z	WBN harboring pHY <i>adh_S:pd_{CZ}</i>	This study
Plasmids	Description	Source
pHY300PLK	Shuttle vector, Tet and Amp resistant	[32]
pHY <i>pd_{CZ}adh_Z</i>	pHY300PLK with <i>pd_C</i> and <i>adhII</i> of <i>Z. mobilis</i>	This study
pHY <i>pd_{CZ}adh_S</i>	pHY300PLK with <i>Z. mobilis pd_C</i> and <i>S. cerevisiae adhI</i>	This study
pHY <i>pd_{CZ}(adh_S)₂</i>	pHY300PLK with <i>Z. mobilis pd_C</i> and two copies of <i>S. cerevisiae adhI</i>	This study
pHY(<i>pd_{CZ}adh_S</i>) ₂	pHY300PLK with two copies of both <i>Z. mobilis pd_C</i> and <i>S. cerevisiae adhI</i>	This study
pHY <i>adh_S:pd_{CZ}</i>	pHY300PLK with <i>adh_S:pd_{CZ}</i> fusion gene	This study
pHY <i>pd_{CZ}:adh_S</i>	pHY300PLK with <i>pd_{CZ}:adh_S</i> fusion gene	This study
pDH88	Integration vector, chloramphenicol resistant	[33]
pDHtldh	pDH88 with a 325 bp fragment from within <i>B. subtilis ldh</i>	This study

Strains	Description	Source
Primers	Sequence	Restriction enzymes
<i>pd_Z</i> -F	ATTGAATTCCATATGAAGGAGGAGTAAGCAATGAGTTATACTGTCCG	<i>EcoRI</i>
<i>pd_Z</i> -R	ATAGGATCCCTAGAGGAGCTTGTTAACAGGCTTACG	<i>BamHI</i>
<i>adh_Z</i> -F	ATAGGATCCAAGGAGGGTATAGCTATGGCTTCTTCAACTTTTTATATTC	<i>BamHI</i>
<i>adh_Z</i> -R	ATATCTAGACATATGTCAGAAAGCGCTCAGGAAGAGTTCTTCAAC	<i>XbaI</i>
<i>adh_S</i> -F	ATGGATCCAAAGGAGGCGATTTGATGTCTATCCCAGAAACTC	<i>BamHI</i>
<i>adh_S</i> -R	ATTCTAGATTAATGATGATGATGATGATGTTTAGAAGTGCAACAAC	<i>XbaI</i>
<i>tldh</i> -F	ATAAAGCTTTCTTACGGAACATATGAAGACTGC	<i>HindIII</i>
<i>tldh</i> -R	ATATCTAGACGTGTACGTTTTGAGGCGC	<i>XbaI</i>
<i>dldh</i> -F	GTAGCTTTAATCGGAGCGGG	-
<i>dldh</i> -R	GCGACATCGTATAACGTTACTGG	-

Construction of ethanologenic operons in pHY300PLK

The molecular biology methods such as restriction digestion, ligation, transformation, and agarose gel electrophoresis were all according to standard protocols [29]. The *pd_C* and *adh* genes were obtained from *Z. mobilis* and *S. cerevisiae* by PCR. For this purpose, the overnight cultures of *Z. mobilis* and *S. cerevisiae* were harvested by centrifugation (12000 ×g, 20 min) and used for DNA extraction using a genomic DNA purification kit (GeneAll, Korea). The purified DNA of each strain was used as the template in PCR reactions with specific primers listed in Table 1. The amplified genes were purified and digested with appropriate restriction enzymes and then ligated into pHY300PLK to make artificial operons using *Z. mobilis**pd_C* and *adh_B* or *Z. mobilis**pd_C* and *S. cerevisiae**adh_I* under the control of *Tet* promoter. Therefore, two recombinant plasmids namely pHY*pd_C*-*adh_Z* and pHY*pd_C*-*adh_S* were constructed (Table 1). In addition, two other plasmids including pHY(*pd_C*-*adh_S*)₂ with an operon containing two copies of both *pd_C* and *adh_S* and the other plasmid pHY*pd_C*-(*adh_S*)₂ with an operon containing one copy of *pd_C* but two copies of *adh_S* were constructed. Also, two fusion genes were synthesized using *Z. mobilis**pd_C* and *S. cerevisiae* *adh* by in-frame cloning of the genes consecutively in pHY300PLK [32]. The genes were fused in alternate orientations while the stop nucleotides of the first gene and the start nucleotides of the second gene were deleted. Therefore, two plasmids namely pHY*pd_C*:*adh_S* and pHY*adh_S*:*pd_C* were constructed (Table 1). The authenticity of the resulting plasmids was confirmed by sequencing (Pishgam Company, Tehran, Iran).

Inactivation of *ldh* in *B. subtilis* WB600

The chromosomal gene encoding lactate dehydrogenase (*ldh*) in *B. subtilis* WB600 was disrupted by homologous recombination. For this purpose, a DNA fragment of 325 bp was amplified from within the gene using primers *tl dh*-F and *tl dh*-R. The amplified fragment (named *tl dh*) was digested with *Hind*III and *Xba*I, and then ligated into pDH88 [33]. The resulting plasmid, pDHT*ldh*, was used for the transformation of *B. subtilis* WB600. Transformants were selected on LB medium containing 5 µg/ml chloramphenicol and checked for a Δ *ldh* strain by PCR with *dldh*-F and *dldh*-R primers and sequencing. The select strain, named WBN, was used for transformation by every constructed plasmid including pHY*pd c_Z-adh_Z*, pHY*pd c_Z-adh_S*, pHY(*pd c_Z-adh_S*)₂, pHY*pd c_Z-(adh_S)₂*, pHY*adh_S;pd c_Z*, and pHY*pd c_Z;adh_S* giving rise to strains NZ, NZS, N(ZS)2, NZS2, NS:Z, and NZ:S, respectively (Table 1).

Fermentation conditions

For ethanol production, *B. subtilis* strains harboring the heterologous ethanologenic operons were cultured overnight at 37 °C in 2YT broth (1.6 % Tryptone, 1 % yeast extract, 0.5 % NaCl, pH 7) containing 5 µg/ml chloramphenicol and 20 µg/ml tetracycline. The growing cells were used as inoculum to an initial optical density (OD_{600 nm}) of 0.1 in one-liter flasks containing 100 ml of 2YT broth supplemented with either glucose or dried ground potatoes (DGP). The DGP was prepared using potatoes obtained from a local market. The potatoes were peeled, washed, and grated before being dried in an oven at 50 °C. The dried biomass was ground using a grinder and used in the preparation of culture mediums for ethanol production at 50, 100, and 150 g/L concentrations. The cultures were conducted at 37 °C and 180 rpm in flasks plugged with cotton balls as normal aeration (NA) conditions. Where limit aeration (LA) was required, the cotton-plugged flasks were covered with aluminum caps. For biphasic cultures, the growing cells were incubated under normal aeration for the first 48 hours and then under limited aeration for another 48 hours. At the end of fermentation, the residual biomass was separated by centrifugation (12000 ×g, 20 min) and dried to obtain the weight.

Analytical methods

The growth was monitored by determination of the optical density of the culture medium at 600 nm. The glucose concentration was measured using a glucose oxidase kit (Pars Azmoon, Tehran, Iran). The ethanol concentration was measured by a Varian CP-3800 gas chromatograph equipped with a CP-Wax 57 CB column, a 1041 injector, and a flame ionization detector (FID). All GC analyses were performed under the same conditions as follows: Helium of high purity as the carrier gas at a flow rate of 1 ml/min, the injection port temperature of 200 °C, and the detector temperature of 250 °C. The ethanol concentration in the culture medium was calculated by a linear regression equation that was prepared using a series of standard ethanol concentrations in water. Methanol was used as an internal standard added into all samples at 2 g/L concentration.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by National Institute of Genetic Engineering and Biotechnology (NIGEB) with grants No. 680 and 762.

Authors' contributions

Maleki, M. Changizian, N. Zolfaghari, and S. Rajaei performed the experiments. K. A. Noghabi, and H. S. Zahiri designed the experiments and supervised the research.

Acknowledgements

Not applicable.

References

1. Dien B, Cotta M, Jeffries T. Bacteria engineered for fuel ethanol production: current status. *Appl Microbiol Biotechnol*. 2003;63(3):258–66.
2. Tian L, Papanek B, Olson DG, Rydzak T, Holwerda EK, Zheng T, Zhou J, Maloney M, Jiang N, Giannone RJ. Simultaneous achievement of high ethanol yield and titer in *Clostridium thermocellum*. *Biotechnol Biofuels*. 2016;9(1):116.
3. Cripwell R, Favaro L, Rose SH, Basaglia M, Cagnin L, Casella S, van Zyl W. Utilisation of wheat bran as a substrate for bioethanol production using recombinant cellulases and amyolytic yeast. *Appl Energy*. 2015;160:610–7.
4. Sakwa L, Cripwell R, Rose S, Viljoen-Bloom M. Consolidated bioprocessing of raw starch with *Saccharomyces cerevisiae* strains expressing fungal alpha-amylase and glucoamylase combinations. *FEMS Yeast Res*. 2018;18(7):foy085.
5. Talarico LA, Gil MA, Yomano LP, Ingram LO, Maupin-Furlow JA. Construction and expression of an ethanol production operon in Gram-positive bacteria. *Microbiology*. 2005;151(12):4023–31.

6. Romero S, Merino E, Bolívar F, Gosset G, Martinez A. Metabolic engineering of *Bacillus subtilis* for ethanol production: lactate dehydrogenase plays a key role in fermentative metabolism. *Appl Environ Microbiol.* 2007;73(16):5190–8.
7. Liu Y, Li J, Du G, Chen J, Liu L. Metabolic engineering of *Bacillus subtilis* fueled by systems biology: Recent advances and future directions. *Biotechnol Adv.* 2017;35(1):20–30.
8. Chen J, Zhao L, Fu G, Zhou W, Sun Y, Zheng P, Sun J, Zhang D. A novel strategy for protein production using non-classical secretion pathway in *Bacillus subtilis*. *Microb Cell Fact.* 2016;15(1):69.
9. Paoletti L, Lu Y-J, Schujman GE, de Mendoza D, Rock CO. Coupling of fatty acid and phospholipid synthesis in *Bacillus subtilis*. *J Bacteriol.* 2007;189(16):5816–24.
10. Sun G, Sharkova E, Chesnut R, Birkey S, Duggan MF, Sorokin A, Pujic P, Ehrlich SD, Hulett FM. Regulators of aerobic and anaerobic respiration in *Bacillus subtilis*. *J Bacteriol.* 1996;178(5):1374–85.
11. Zhang XZ, Zhang YHP. One-step production of biocommodities from lignocellulosic biomass by recombinant cellulolytic *Bacillus subtilis*: Opportunities and challenges. *Eng Life Sci.* 2010;10(5):398–406.
12. Stülke J, Hillen W. Regulation of carbon catabolism in *Bacillus* species. *Annu Rev Microbiol.* 2000;54(1):849–80.
13. Gu Y, Xu X, Wu Y, Niu T, Liu Y, Li J, Du G, Liu L. Advances and prospects of *Bacillus subtilis* cellular factories: from rational design to industrial applications. *Metab Eng.* 2018;50:109–21.
14. Banerjee S, Mishra G, Roy A. Metabolic Engineering of Bacteria for Renewable Bioethanol Production from Cellulosic Biomass. *Biotechnol Bioprocess Eng.* 2019;24:713–33.
15. Awasthi D, Wang L, Rhee MS, Wang Q, Chauliac D, Ingram LO, Shanmugam KT. Metabolic engineering of *Bacillus subtilis* for production of D-lactic acid. *Biotechnol Bioeng.* 2018;115(2):453–63.
16. Yang T, Rao Z, Hu G, Zhang X, Liu M, Dai Y, Xu M, Xu Z, Yang S-T. Metabolic engineering of *Bacillus subtilis* for redistributing the carbon flux to 2, 3-butanediol by manipulating NADH levels. *Biotechnol Biofuels.* 2015;8(1):129.
17. Schönert S, Seitz S, Krafft H, Feuerbaum E-A, Andernach I, Witz G, Dahl MK. Maltose and maltodextrin utilization by *Bacillus subtilis*. *J Bacteriol.* 2006;188(11):3911–22.
18. Deng Y, Olson DG, Zhou J, Herring CD, Shaw AJ, Lynd LR. Redirecting carbon flux through exogenous pyruvate kinase to achieve high ethanol yields in *Clostridium thermocellum*. *Metab Eng.* 2013;15:151–8.
19. Barbosa MF, Ingram L. Expression of the *Zymomonas mobilis* alcohol dehydrogenase II (*adhB*) and pyruvate decarboxylase (*pdC*) genes in *Bacillus*. *Curr Microbiol.* 1994;28(5):279–82.
20. Gold RS, Meagher MM, Tong S, Hutkins RW, Conway T. Cloning and expression of the *Zymomonas mobilis* “production of ethanol” genes in *Lactobacillus casei*. *Curr Microbiol.* 1996;33(4):256–60.
21. Inui M, Kawaguchi H, Murakami S, Vertès AA, Yukawa H. Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. *J Mol Microbiol Biotechnol.* 2005;8(4):243–54.

22. Liu S, Nichols NN, Dien BS, Cotta MA. Metabolic engineering of a *Lactobacillus plantarum* double *ldh* knockout strain for enhanced ethanol production. *J Ind Microbiol Biotechnol*. 2006;33(1):1–7.
23. Nichols NN, Dien BS, Bothast RJ. Engineering lactic acid bacteria with pyruvate decarboxylase and alcohol dehydrogenase genes for ethanol production from *Zymomonas mobilis*. *J Ind Microbiol Biotechnol*. 2003;30(5):315–21.
24. Ramos HC, Hoffmann T, Marino M, Nedjari H, Presecan-Siedel E, Dreesen O, Glaser P, Jahn D. Fermentative metabolism of *Bacillus subtilis*: physiology and regulation of gene expression. *J Bacteriol*. 2000;182(11):3072–80.
25. Lewicka AJ, Lyczakowski JJ, Blackhurst G, Pashkuleva C, Rothschild-Mancinelli K, Tautvaišas D, Thornton H, Villanueva H, Xiao W, Slikas J. Fusion of pyruvate decarboxylase and alcohol dehydrogenase increases ethanol production in *Escherichia coli*. *ACS Synth Biol*. 2014;3(12):976–8.
26. Aalbers FS, Fraaije MW. Enzyme fusions in biocatalysis: coupling reactions by pairing enzymes. *ChemBioChem*. 2019;20(1):20–8.
27. Yang H, Liu L, Xu F. The promises and challenges of fusion constructs in protein biochemistry and enzymology. *Appl Microbiol Biotechnol*. 2016;100(19):8273–81.
28. Ghadikolaie KK, Noghabi KA, Zahiri HS. Development of a bifunctional xylanase-cellulase chimera with enhanced activity on rice and barley straws using a modular xylanase and an endoglucanase procured from camel rumen metagenome. *Appl Microbiol Biotechnol*. 2017;101(18):6929–39.
29. Green MR, Sambrook J. *Molecular cloning: A Laboratory Manual*, fourth edn. New York: Cold Spring Harbor Laboratory; 2012.
30. Anagnostopoulos C, Spizizen J. Requirements for transformation in *Bacillus subtilis*. *J Bacteriol*. 1961;81(5):741–6.
31. Wu X-C, Lee W, Tran L, Wong S. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J Bacteriol*. 1991;173(16):4952–8.
32. Ishiwa H, Shibahara H. New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*. II. Plasmid pHY300PLK, a multipurpose cloning vector with a polylinker, derived from pHY460. *Jpn J Genet* 1985; 60:235–243.
33. Henner DJ: Inducible expression of regulatory genes in *Bacillus subtilis*. In: *Methods Enzymol*. vol. 185: Elsevier; 1990: 223–228.

Figures

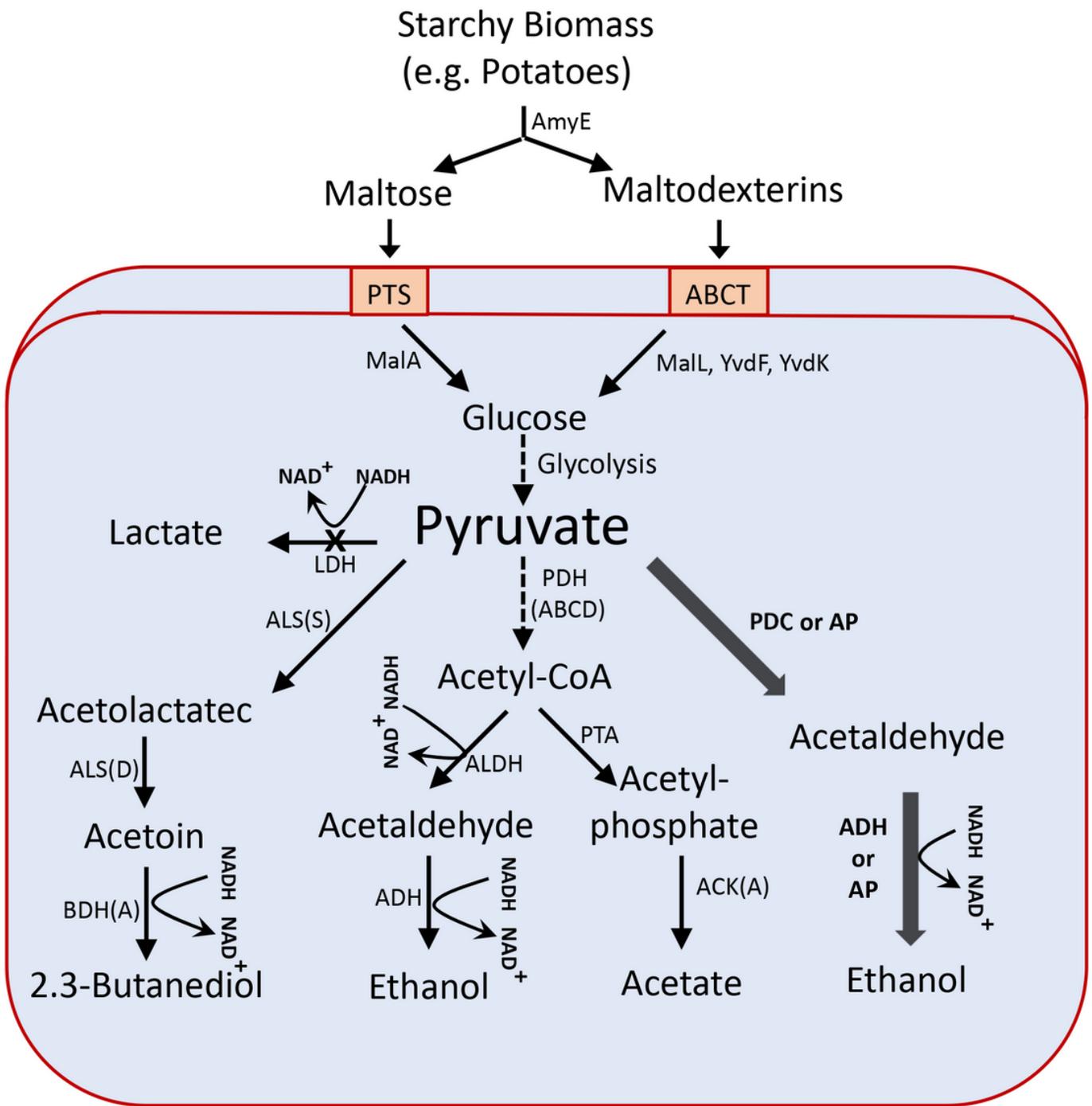


Figure 1

Schematic representation of *B. subtilis* fermentation metabolism and ethanol production from potatoes using the engineered heterologous pathways. The thin arrows indicate the intrinsic metabolic pathways and the thick arrows point to the engineered pathway composed of either PDC and ADH or the bifunctional AP fusion. AmyE, extracellular amylase; PTS, phosphotransferase system; MalA, phospho- α -1,4-glucosidase; ABCT, ABC transporter; YvdF, maltogenic amylase; YvdK, maltose phosphorylase; MalL, glucosidase; LDH, lactate dehydrogenase; ALS, acetolactate synthase; BDH, butanediol dehydrogenase; PDH, pyruvate dehydrogenase; ALDH, acetaldehyde dehydrogenase; PTA, phosphotransacetylase; ACK, acetate kinase.

acetate kinase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; AP, bifunctional AHD:PDC fusion.

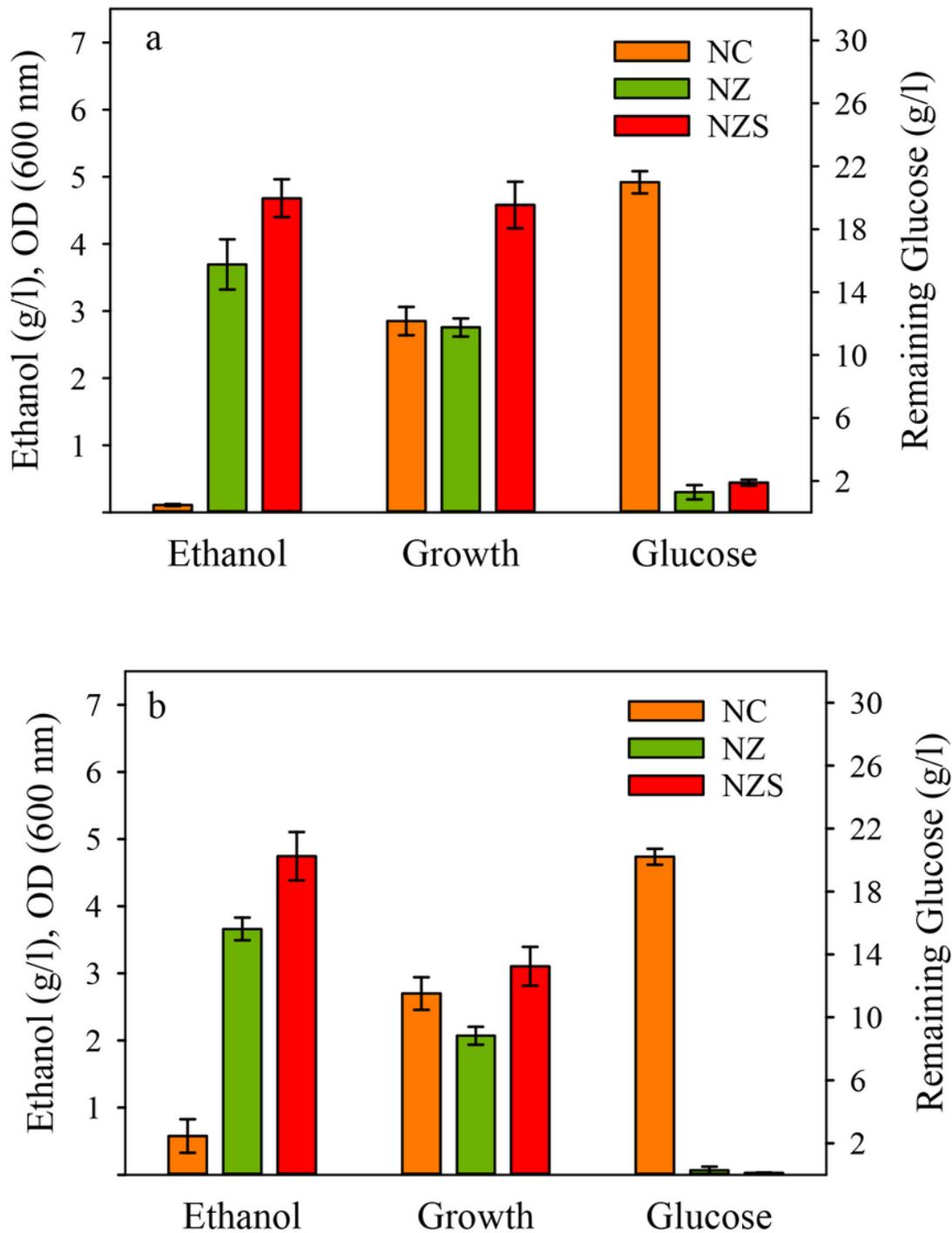


Figure 2

Ethanol production using strains NZ (Δ ldh, pdcZ-adhZ) and NZS (Δ ldh, pdcZ-adhS). The strains along with the control strain NC (Δ ldh) were cultured in 2YT medium containing 40 g/L glucose at 37 °C, and 180 rpm for 96 h under limited aeration conditions. Samples were taken at (a) 48 hours, and (b) 96 hours of fermentation, and analyzed for ethanol production, cell growth, and glucose consumption.

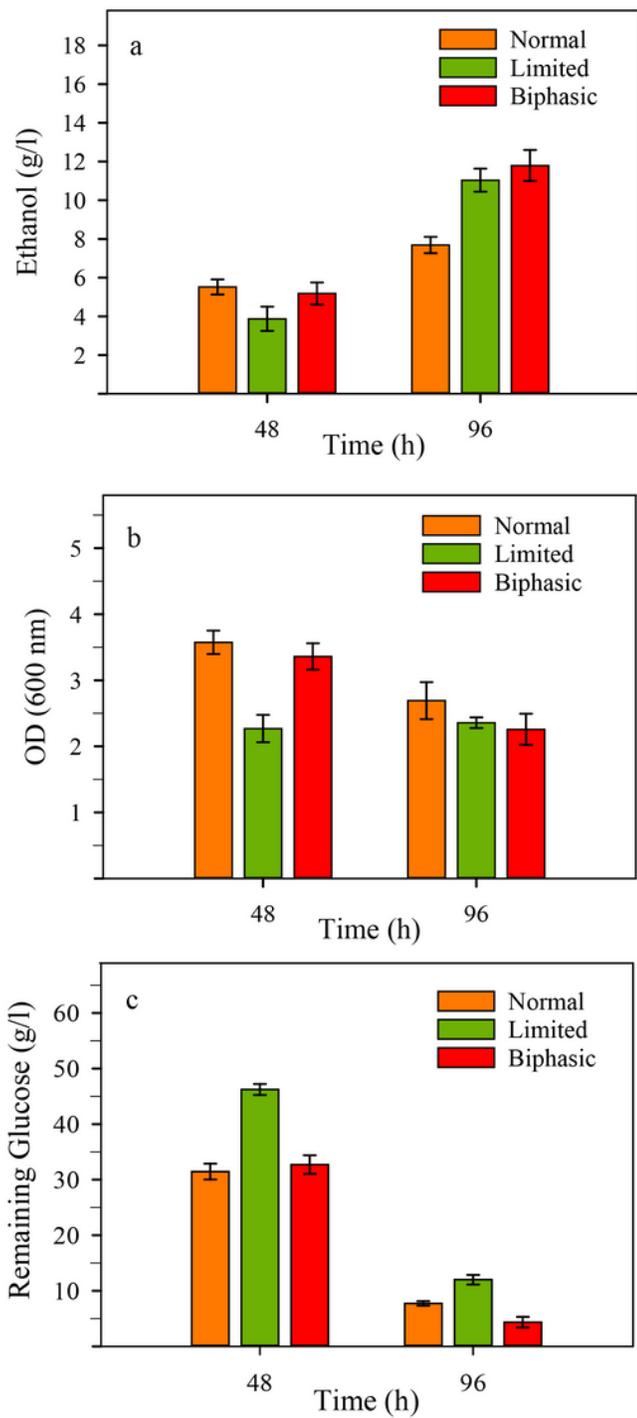


Figure 3

Ethanol production using *B. subtilis* NZS (Δ ldh, pdcZ-adhS) under various aeration conditions. Cultures were conducted for 96 h in a shaking incubator at 37 °C and 180 rpm under the normal, limited, and biphasic aeration conditions. Samples were taken at 48 hours and 96 hours of fermentation and analyzed for: (a) ethanol production, (b) cell growth, and (c) glucose consumption.

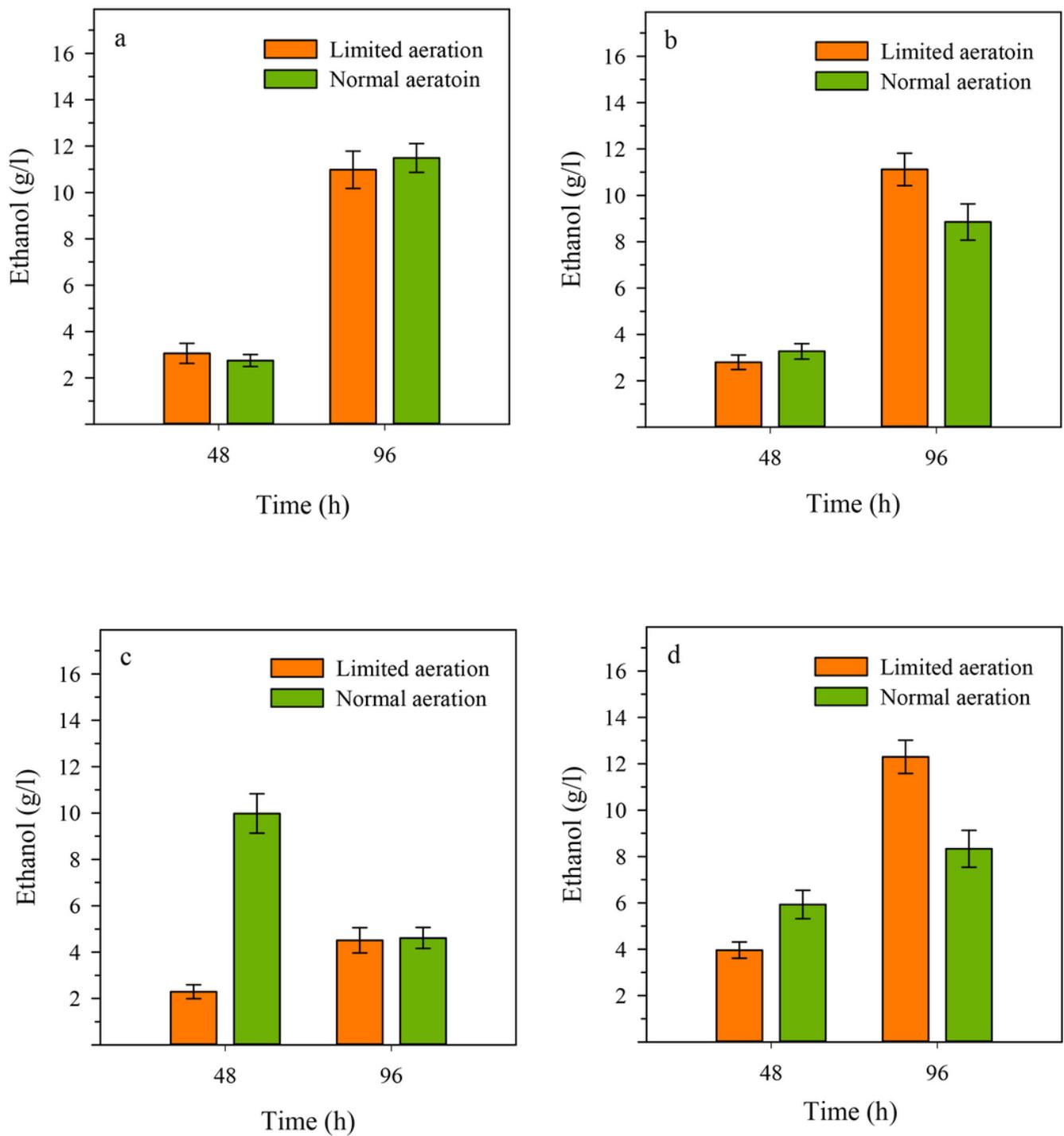


Figure 4

The combinatorial influence of aeration, temperature, and shaking conditions on ethanol production by strain NZS (Δ ldh, pdcZ-adhS). Cultures with limited and normal aeration were incubated at: (a) 30 °C, 120 rpm; (b) 30 °C, 180 rpm; (c) 37 °C, 120 rpm; and (d) 37 °C, 180 rpm. Samples obtained at 48 hours and 96 hours of fermentation were analyzed for ethanol concentration.

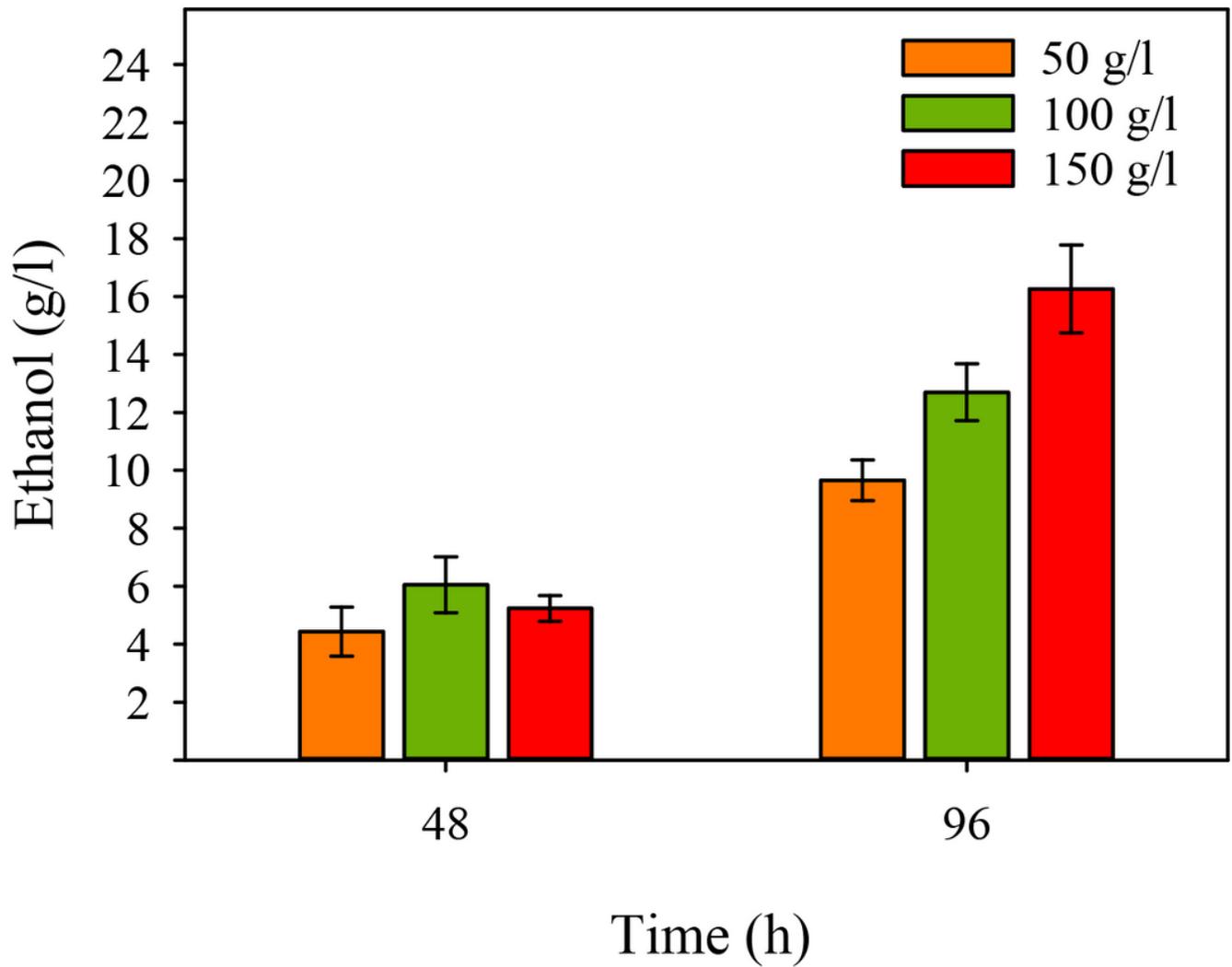


Figure 5

CBP ethanol production by strain NZS (Δ ldh, pdcZ-adhS) using potatoes as a typical starchy biomass. Cultures were conducted in 2YT medium supplemented with 50, 100, and 150 g/L dried ground potatoes (DGP) at 37 °C and 180 rpm under limited aeration using a tiny inoculum (OD₆₀₀ 0.1) of NZS. Samples, taken after 48 hours and 96 hours, were analyzed for ethanol concentration in culture mediums.

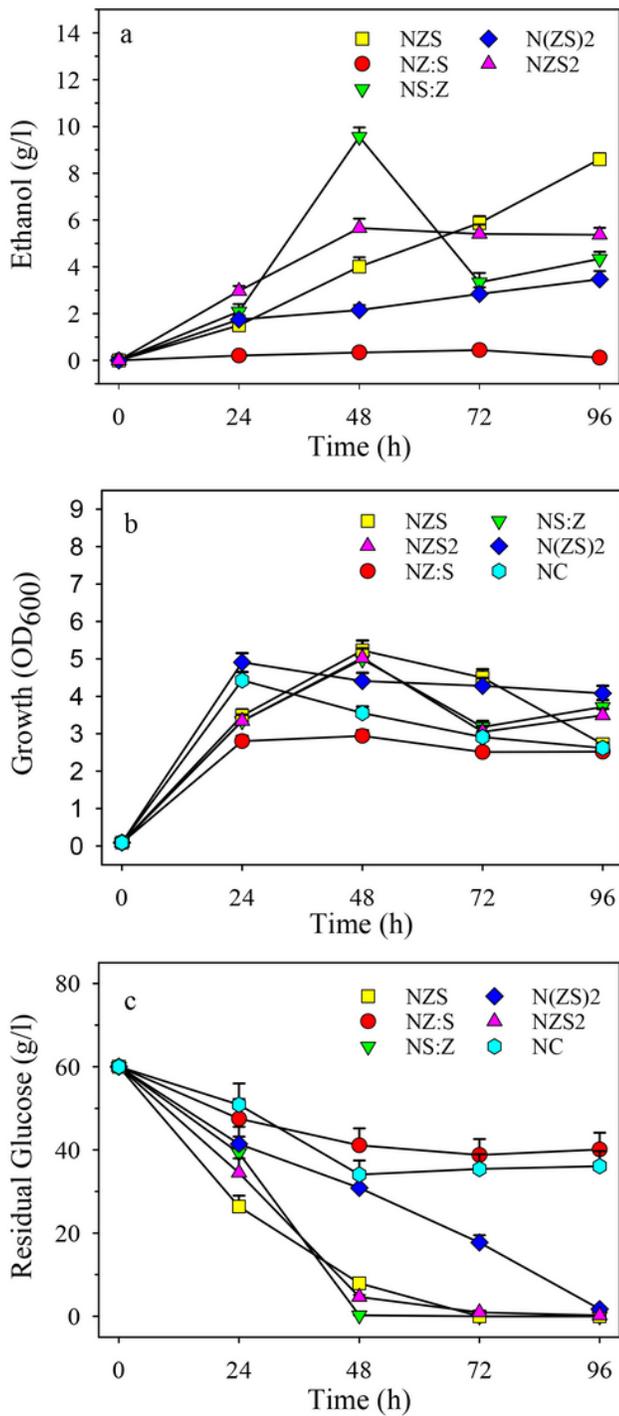


Figure 6

Ethanol production by *B. subtilis* strains harboring different numbers of *pdhZ* and *adhS* as well as fusions of the genes. Strains NZS (Δ ldh, *pdhZ*-*adhS*), N(ZS)2 (Δ ldh, (*pdhZ*-*adhS*)₂), NZS2 (Δ ldh, *pdhZ*-(*adhS*)₂), NZ:S (Δ ldh, *pdhZ*:*adhS*), and NS:Z (Δ ldh, *adhS*:*pdhZ*) containing, respectively, one copy of each gene, two copies of each gene, one *pdhZ* but two *adhS*, *adhS* fused to *pdhZ*, and *pdhZ* fused to *adhS*, were cultured with an initial optical density of 0.1 (600 nm) in 2YT mediums supplemented with 60 g/L glucose and

incubated at 37 °C, 180 rpm. Samples taken at 24-hour intervals were analyzed for: (a) ethanol production, (b) cell growth, and (c) glucose consumption.

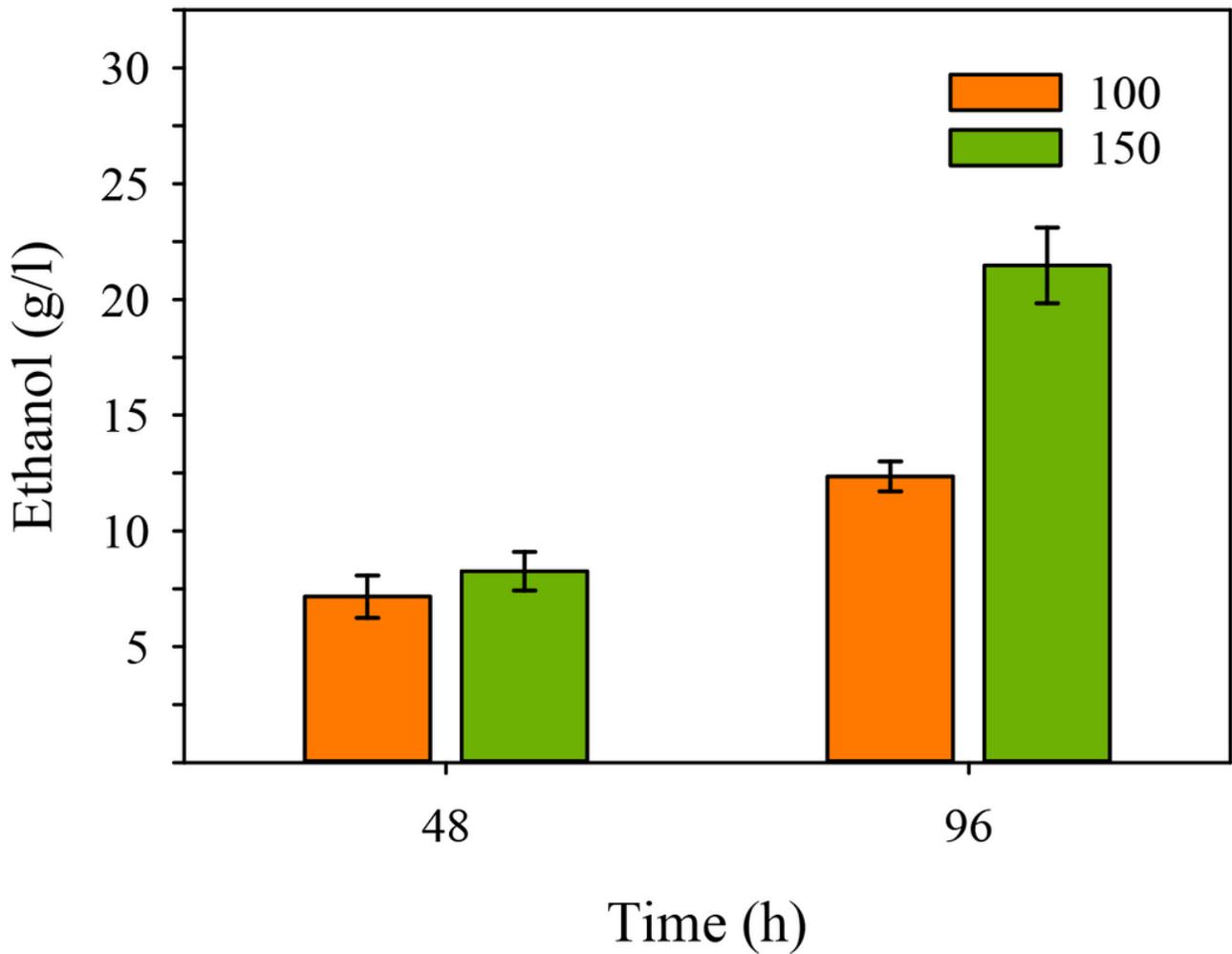


Figure 7

CBP ethanol production by strain NS:Z (Δ ldh, adhS:pdz). Cultures were conducted in 2YT medium supplemented with 100 and 150 g/L dried ground potatoes (DGP) at 37 °C, 180 rpm under limited aeration conditions.