Fermentation performance of Bacillus subtilis wild type and alsS-mutant using different carbon sources

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Abstract

Bacillus subtilis can metabolize a wide array of sugars under aerobic conditions, including hexoses via the EMP pathway and some pentoses via the pentose phosphate pathway, and secretes amylases, cellulases and hemicellulases. However, studies using different sugars under anaerobic conditions are scarce. *B. subtilis* can also grow anaerobically by using a terminal electron acceptor or by fermentation metabolizing glucose to lactic acid (LA), butanediol, and other products under non-aerated conditions. *B. subtilis* ER382 (Δ*alsS*), with the 2,3-butanediol pathway inactivated, was obtained from strain 168 trp^+. Such deletion allowed obtaining a homo l-lactogenic strain with increased LA production. *B. subtilis* ER382 was evaluated in batch cultures with 10 g/L of seven different carbon sources under non-aerated conditions and rich media. *B. subtilis* ER382 metabolized glucose, fructose, cellobiose, sucrose, and arabinose under non-aerated conditions and produced LA as the only fermentation product. However, strains 168 trp^+ and ER382 were unable to ferment and grow on glycerol or starch under the tested conditions. The higher biomass yield and faster rates of sugar consumption (1.01 g_{SUGAR}/L h) and L-LA production (1.32 g_{LA}/L h) were obtained with glucose as the carbon source and the strain containing the *alsS* deletion. The L-LA yield on consumed sugars was above the theoretical because carbon sources from yeast extract and amino acids from casein hydrolysate were also used for LA synthesis.

Introduction

About 90% of the total lactic acid (LA) in the world is produced by microbial fermentation mainly from glucose (Komesu et al., 2017; Martinez et al., 2017). Although having complex nutrient requirements (Abdel-Rahman et al., 2013; Abedi and Hashemi, 2020), *Lactobacillus* are the main microorganisms used to produce acid lactic, primary *delbrueckii.* Also, other wild type or metabolically engineered microorganisms can be used to produce LA, i.e., *Rhizopus*, *Escherichia*, *Kluyveromyces*, *Saccharomyces*, and *Bacillus* strains (Okano et al., 2010; Abdel-Rahman et al., 2013; Idler et al., 2015; Martinez et al., 2017; Gu et al., 2018; Abedi and Hashemi, 2020). There are numerous reports on LA production by some *Bacillus* species, like *Bacillus coagulans* (Wang et al., 2014; Xu *et al*., 2014; Ong et al., 2016; Cubas-Cano et al., 2020; Azaizeh et al., 2020). Furthermore, *Bacillus subtilis* also produces LA. *B. subtilis* is Generally Recognized as Safe (GRAS), and it has been reported that under aerobic conditions, *B. subtilis* can metabolize a wide array of sugars (Steinmetz, 1993), secretes amylases, cellulases and hemicellulases, and can metabolize hexoses via the EMP pathway and some pentoses via the pentose phosphate pathway. *B. subtilis* can also grow anaerobically by using a terminal electron acceptor or fermentation and can ferment glucose to the enantiomer L-lactic acid (LA), butanediol, and other products under non-aerated conditions (Nakano et al., 1997 and 1998).

Few studies on growth performance, carbon source consumption, and homolactic acid production using different carbon sources under non-aerated conditions have been reported. Romero *et al.* (2009) reported that 105 g/L LA could be produced by *B. subtilis* CHI *als*S^- (a prototroph and proteases deficient strain obtained from *B. subtilis* W700) from 10% glucose in a very long term fermentation (216 h) in the mineral medium; Gao et al. (2012) showed that a *B. subtilis* mutant named MUR1 (a UV mutant of *B. subtilis* 1A304 (Ø105MU331)), could produce 99.3 and 183.2 g/L of LA in 12 and 52 h, respectively, with a 98.5% substrate conversion yield and a maximum LA production rate of 16.1 g/(L h) from complex medium supplemented with glucose and using fed-batch cultures. Recently, Awasthi et al. (2018) reported the production of d-LA at 48°C using the metabolically engineered *B. subtilis* strain DA12 (HB1000 Δ*ldh* Δ*als ldh*A^+, a derivative of strain 168), in a mineral medium supplemented with 30 g/L glucose at a yield of 0.89 g/g glucose and the D-lactate titer was 16.2 g/L in 168 h.

Overall, these results show that *B. subtilis* can efficiently ferment glucose to LA. This study aimed to generate a homolactic *B. subtilis* strain by deleting the gene encoding for the acetolactate synthase (*alsS*) to eliminate the formation of 2,3 butanediol and to evaluate the consumption and fermentation of several carbon sources that this bacterium consumes under aerobic conditions. Carbon sources included hexoses (glucose, fructose), pentose (arabinose), and disaccharides (cellobiose, sucrose), glycerol, and a polysaccharide (starch).

Materials And Methods

Construction of the *B. subtilis* Δ*als*S: strain ER382

The strains, plasmids, and oligonucleotides used in this study are listed in Tables 1, 2 and 3. Standard procedures for plasmid preparation, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis were used (Sambrook *et al*., 1992). *E. coli* XL1-Blue was used as a host for the construction of plasmids. Cells were cultured in Luria-Bertani medium (per liter: 10 g tryptone; 5 g yeast extract; 5 g NaCl). *B. subtilis* was transformed using the natural competition method (Cutting & Vander-Horn, 1990). Chromosomal integrations and deletions were confirmed using appropriate antibiotic markers, PCR analysis, and quantification of fermentation products.

Cloning and inactivation of the alsS gene. The spectinomycin cassette flanked by *lox*P sites was amplified by PCR from plasmid pLoxSpec-Walt (Table 1) using the oligonucleotides SpecR_up and SpecR-down (Table 3). This DNA segment was cloned in plasmid pJET-1.2 to

obtain vector pJET::*lx*Spt*lx*. The vector pTOPO*als*S (Romero *et al*., 2007) was digested with the restriction enzyme HpaI, leaving blunt sites, while the vector pJET::*lx*Spt*lx* was digested in the XhoI and NcoI sites (obtaining cohesive sites), to cleave the spectinomycin cassette with the *lox*P site. Subsequently, this DNA fragment was polymerized with the T4_DNA polymerase (Biolabs Inc. MA, US) to obtain blunt sites. The ligation of the fragment *lx*Spt*lx* (*lox*P-Spectinomycin-*lox*P) in the linearized vector pTOPO*als*S was carried out with T4_DNA ligase (Biolabs Inc. MA, US) to obtain pTOPOalsS::*lx*Spt*lx*. *B. subtilis* 168 trp$^+$ was transformed, by natural competition, with the linear DNA fragment (alsS-*lx*Spt*lx*P'-alsS') obtained from the vector pTOPOals::*lx*Spt*lx* with oligonucleotides alsDinicio and alsS (Table 3). The integration into the chromosome was confirmed using spectinomycin (100 µg/mL) to select the *B. subtilis* strain 168 trp$^+$ (Δ*als*S::*lx*Spt*lx*). Subsequently, this strain was transformed with the plasmid pCRM-Ts-Phleo (Table 2) to remove the spectinomycin cassette. This plasmid contains the gene encoding a CRE recombinase, which recognizes the sequence of the *lox*P sites and performs double homologous recombination, which in this case excises the spectinomycin cassette generating only one *loxP* site. The *B. subtilis* strain ER382 (*B. subtilis* 168 trp$^+$ Δ*als*S) was obtained after selecting colonies in the absence of spectinomycin and confirmed by PCR with oligonucleotides alsDinicio and alsS.

Inoculum preparation and growing conditions

Strains from frozen vials were plated in Petri dishes with solid Luria Bertani medium and incubated overnight at 37°C. Cells from plates were used to inoculate a 250 ml flask with 150 mL of the same cultivation media and incubated overnight at 37°C, 120 rpm. Cells from the flasks were centrifuged and utilized as inoculum to obtain an initial optical density of 0.05 at 600 nm (OD_{600}). Non-aerated cultivations were performed in 250-mL mini-fermenters with a working volume of 200 mL, under non-aerated conditions at 37°C, pH 7.0 (controlled by the automatic addition of 2N KOH), and 100 rpm (Beall *et al*., 1991). *B. subtilis* grows deficiently on mineral media under non-aerated conditions requiring the addition of pyruvate or tryptophan (Nakano et al, 1998; Romero et al, 2009), hence the strains were cultured in Luria-Bertani medium plus 10 g of the different carbon sources: glucose, fructose, cellobiose, sucrose, arabinose, glycerol, or starch. Each experiment was performed in duplicate, and the data reported in the graphs show the average and the standard error. Samples from fermentations (1 mL each) were centrifuged (2800 x g, 5 min, room temperature). The supernatants were stored at -70°C until their analysis.

Analytical methods

Optical density was measured in a spectrophotometer (Perkin Elmer Lambda 11, USA) and converted to dry cell weight (DWC) using a calibration curve, 1-DO 600 nm = 0.35 g_{DWC}/L (Martinez *et al*., 1997). The quantification of sugars, glycerol, lactic acid and 2,3-butanediol was performed by HPLC (Model 996, Waters, Millipore Co., Milford, MA, USA) equipped with UV and refractive index detectors. The separations were carried out at 50°C, using an Aminex HPX-87H ion exclusion column (300 × 7.8 mm; BioRad Laboratories, Hercules, CA), 5 mM H_2SO_4 as mobile phase, a flow rate of 0.5 mL/min and an injection volume of 20 µL of each sample or calibration standard. To confirm the generation of the l stereoisomer, l-Lactate was determined in a biochemical analyzer (YSI Model 2700, YSI Inc., Yellow Springs, OH), using immobilized l-lactate oxidase.

Results

Characterization of *B. subtilis* strains 168 trp$^+$ and ER382 (Δ*als*S) with glucose as carbon source.

Fermentation of glucose by *B. subtilis* 168 trp$^+$ (parent strain) and *B. subtilis* ER382 (Δ*als*S) in Luria medium supplemented with 10 g/L of glucose is shown in Figure 1. The fermentation parameters are shown in Table 4. The amount of biomass formed at glucose depletion with strain ER382 was 52 % higher than for *B. subtilis* 168 trp$^+$. The glucose was consumed 1.4-times faster by the strain ER382 than the parent strain, depleting the sugar at 10 h of fermentation, while strain 168 trp$^+$ depleted the glucose after 14 h. The progenitor strain reached 8.78 g/L of LA, producing 2.43 g/L of 2,3-butanediol. But, 2,3-butanediol production was abolished in ER382, reaching 13.2 g/L of LA. Other fermentation products were not found by HPLC, hence the deletion of *alsS* allowed to generate a homo l-lactogenic strain. This value indicates that the components of the Luria-Bertani medium (i.e., the amino acids) also provided carbon for the formation of LA, allowing to produce 32% more than the theoretical yield from glucose (1 g_{LA}/1 g of consumed glucose). Concomitantly to the increase in the rate of volumetric glucose consumption, the volumetric LA productivity was 2.1-times higher with the ER382 strain compared to the parent strain.

Evaluation of *B. subtilis* 168 trp$^+$ and *B. subtilis* ER382 with fructose, sucrose, cellobiose, or arabinose as carbon sources.

Both strains, 168 trp$^+$ and ER382, were able to grow and ferment fructose (Figure 2), cellobiose (Figure 3a), sucrose (Figure 3b), or arabinose (Figure 3c). However, both strains could not ferment glycerol or starch (data not shown). For glycerol as a carbon source,

probably the strains needed a final electron acceptor to be metabolized under anaerobic conditions (Nakano *et al*., 1998). It is well known that *B. subtilis* can metabolize starch under aerobic conditions. We suggest that under non-aerated conditions, *B. subtilis* 168 may not be expressing the genes necessary to synthesize the enzymes that hydrolyze starch. Also, it is known that *B. subtilis* cannot metabolize lactose and xylose because of the lack of specific transport systems (Stülken and Hillen 2000).

Fructose The mutant strain ER382 was able to grow under non-aerated conditions using fructose as a carbon source (Figure 2). The yield of biomass was 0.087 g_X/g_S. In addition, the ER382 strain produced 12.9 g/L of LA as the only fermentation product, i.e., 29% more than the theoretical yield (Table 4), with a volumetric LA productivity 18% lower than that obtained when consuming glucose.

Cellobiose No defined exponential growth was observed when cellobiose, sucrose, and arabinose were used as carbon sources (Figure 3 a, b, and c). Therefore, for comparison purposes, the fermentation performance was carried out using the volumetric rates of sugars consumption, the volumetric LA productivity, and the concentration of fermentation products and biomass formation at the end of cultivations. The amount of biomass and volumetric LA productivity reached was almost 15% higher in the ER382 strain when compared to 168 trp^+, exhausting the cellobiose after 50 h of fermentation. The progenitor and the Δ*als*S strain produced 12.2 and 12.5 g/L of LA, respectively. Additionally, the parental strain produced 1.40 g/L of 2,3-butanediol, while the mutant strain had LA as the only fermentation product. Comparing the performance of the parent strain using glucose or cellobiose as carbon sources, it can be observed that the LA yield was slightly higher using cellobiose concomitantly with a lower production of 2,3-butanediol (Table 4). This result indicates that carbon sources affect the regulation of fermentation pathways.

Sucrose When sucrose was used as the carbon source, the biomass was 33% higher with ER382 than the parent strain; however, the LA volumetric productivity was the same with both strains, and LA formation was similar (Table 4, Figure 4b). However, in the mutant strain, no formation of 2,3-butanediol was detected, while the wild strain produced 1.67 g/L. The volumetric sucrose consumption rate was, on average, 20% lower than the rate of cellobiose consumption for both strains.

Arabinose The biomass generated was 55% higher for ER382 (0.84 vs. 0.54 g/L), and the volumetric LA productivity was 30% higher than the parental strain (Table 4). Nevertheless, none strain consumed the sugar completely at 65 h of fermentation. The culture with the wild strain showed a residual arabinose concentration of 3.67 g/L, while the culture with the mutant strain had a residual concentration of 1.14 g/L. The concentration of LA only reached titers of 6.81 and 8.43 g/L with the wild strain and the mutant strain, respectively (Figure 4c, Table 4). The LA yield was higher with the 168 trp^+ strain when compared to the Δ*alsS* strain. These results corroborate that some LA was produced from the components of the LB medium and not from the sugar sources, which causes an apparent increase in the yield.

Summarizing, with the ER382 mutant the biomass generated from the catabolism of disaccharides, hexoses, and pentose was higher when compared to the progenitor strain. In addition, the volumetric sugar consumption rate and volumetric LA productivity were higher when glucose was used compared to the other carbon sources for both strains (Table 4).

Discussion

There are several reports on using different carbon sources with *B. subtilis* under aerobic conditions (among many others: Cabrera-Valladares *et al*., 2012; Ludwing *et al.,* 2001; Blencke *et al*., 2003, Schmiedel *et al*., 1996). However, the reports using different sugars under anaerobic conditions are scarce (Steinmetz 1993; Nakano *et al*., 1997 and 1998; Cruz-Ramos *et al*., 2000; Stülken and Hillen, 2000; Romero *et al*., 2009; Härtig and Jahn, 2012). To evaluate *B. subtilis* 168 trp^+ and ER382 (Δ*alsS*) performance to ferment different carbon sources we decided to use a rich medium, i.e., Luria Bertani, because it has been shown that *B. subtilis* has very poor growth when mineral media-glucose is used under non-aerated conditions (Nakano *et al.,* 1997 and 1998; Cruz-Ramos *et al*., 2000). Under such growth conditions, *B. subtilis* requires the contribution of metabolic intermediates, such as pyruvate (Nakano *et al*., 1997 and 1998; Cruz-Ramos *et al*., 2000), or additional nutrients such as vitamins and/or amino acids, which are provided in a rich medium. Also, to avoid potential interferences, due to the deletion of several proteases and presence of many antibiotic markers, we decided in this study to generate the strain Δ*alsS* from the wild type *B. subtilis* 168 trp^+, instead of the *B. subtilis* strain CHI *alsS* previously developed by Romero *et al*., 2009.

The depletion of oxygen in *B. subtilis* is sensed by the transcriptional regulator FNR and the two-component regulatory system ResDE, which regulates the transcription of several genes, such as *ltcE* (encoding for lactate dehydrogenase) and *als*SD that encodes for the acetolactate synthase and acetolactate decarboxylase, which catalyze the formation of acetoin (precursor of butanediol) (Härtig and Jahn, 2012). Under anaerobic conditions, ATP is generated at the substrate level, and NAD^+ is regenerated in the conversion of pyruvate to lactate and butanediol; this maintains the redox balance and ATP generation for biosynthesis (Nakano *et al*., 1997 and 1998; Steinmetz, 1993; Cruz-Ramos *et al*., 2000; Stülken and Hillen, 2000; Härtig and Jahn, 2012). The results shown in Table 4 suggest that the metabolic balance was maintained when hexoses and disaccharides were used as a carbon source because the LA yield was higher than the theoretical. On

the other hand, to conserve cellular resources, the expression of more than a hundred genes encoding for carbohydrate catabolism activities is induced only when the corresponding carbohydrate is present in the growth medium (Stülken and Hillen, 2000). In *B. subtilis*, this induction can occur via inducer-mediated inactivation of a repressor but can also occur by transcriptional activation or antitermination (Deutscher *et al.,* 2002 and 2014; Fujita, 2009). The internalization of some sugars depends on the phosphoenolpyruvate system (PEP): phosphotransferase of sugars (PTS), a complex enzymatic system responsible for detecting transmembrane transport and phosphorylation of sugar substrates. While few sugars use another type of transport called ABC (Deutscher *et al*., 2002 and 2014; Deutscher, 2008; Fujita, 2009). The PTS is considered a bifunctional system that serves both sugar transport and signal transduction purposes. The regulatory signal senses the inducers or the levels of other important metabolites, like fructose-1,6-biphosphate (FBP) or branched amino acids, to redirect the use of carbon sources to maximize energy requirements (Sonenshein, 2007).

It has been reported that elimination of *als*S does not negatively affect *B. subtilis* growth under anaerobic conditions (Cruz-Ramos et al., 2000; Renna et al., 1993; Frädrich et al., 2012). Accordingly, in comparison to the progenitor strain in ER382 the deletion of *als*S allowed a slight increase in the amount of biomass formed and the biomass yield, the elimination of butanediol formation, the increase of the LA yield, the volumetric sugar consumption rate, and volumetric LA productivity (table 4) Overall, these results indicate that a higher fraction of carbon flux was redirected towards higher biomass and LA formation. Also, the components of the rich media contributed to the formation of LA.

B. subtilis preferentially uses glucose over other carbon sources. This study observed that *B. subtilis* could grow on glucose, fructose, and the capacity to metabolize these hexoses to LA by ER382 under anaerobic conditions was fast, above 1 g_{LA}/L h (Table 4). In *B. subtilis*, glucose is transported and phosphorylated by a PTS-dependent glucose-specific component. The internalization, phosphorylation, and catabolism to pyruvate (glycolysis) generate a low ATP consumption, resulting in a global yield of 2 mol ATP for each mol when glucose is catabolized to LA and restores the NAD^+ balance. It is also known that fructose is transported and phosphorylated via FruA (a PTS fructose transporter) at a high rate (Stülken and Hillen, 2000; Deutsher *et al.,* 2002 and 2014; Fujita, 2009). Compared to glucose, fructose was metabolized at a lower rate because fewer cells were generated using this carbon source (Table 4); the biomass yield on sugar consumed was 33% lower with fructose. Perhaps, the fructose-specific PTS component has a lower transport capacity when compared to that for glucose.

Five genes encoding for beta-glycoside-specific PTS components and a putative 6-phospho-beta-glucosidase (*lic*BCAH) are involved in utilizing beta-glycosidic compounds in *B. subtilis*. The *lic* operon is inducible by lichenan, lichenan hydrolysate, and cellobiose. Furthermore, the expression of the *lic* operon is positively controlled by the LicR regulator protein (Tobisch *et al*., 1999). Since cellobiose is a glucose dimer, knowing that *B. subtilis* has a β-glucosidase and that it requires two ATPs to phosphorylate the two glucoses obtained from cellobiose hydrolysis, it is suggested that this microorganism can use and metabolize this sugar with glucose-like efficiency. However, the rate of cellobiose consumption for both strains was 67–77% lower than the rate at which glucose was consumed. Previously, Chang *et al*. (1994, 1999) and Romero *et al*. (2009) had reported that the use of high concentration of biomass, obtained from aerobic conditions with cellobiose, to inoculate cultures under anaerobic conditions contribute to obtaining cellobiose consumption rates like those observed with glucose due to a greater induction in the genes that are required for cellobiose catabolism.

The presence of sucrose in the medium induces the transcription of genes involved in the regulation, internalization, and metabolism of this carbohydrate. *B. subtilis* has two systems for internalizing this disaccharide into the cell; one that is involved in catabolism is composed of a PTS-permease (*sac*P) and a phosphosucrase (*sac*A) (Débarbouillé *et al.,* 1990; Débarbouillé *et al*., 1991; Crutz and Steinmetz *et al*., 1992; Daguer *et al*., 2004). Under aerobic conditions, the genes for the PTSpermease can be activated at low concentrations of sugar. The second system is mainly involved in anabolism, it is composed of the extracellular enzyme levansucrase (encoded by the *sac*B gene), and under aerobic conditions is more dependent on its activation at high concentrations of sucrose (Débarbouillé *et al*., 1990; Débarbouillé *et al*., 1991; Crutz and Steinmetz *et al*., 1992; Daguer *et al*., 2004). No levan or fructose formation was detected in culture supernatants; hence we suggest that internalization occurred through the SacP transport system. The lower rate of substrate consumption compared to that observed when the carbon source was glucose might have three explanations. First, the activation of the genes for the inducer (sucrose) was low. Although it is known that when only one carbon source is present, the induction of the genes encoding its own transporters must be maximum. However, PTS mediates the autoregulation of carbohydrate utilization by CcpA (a global regulator) (Gunnewijk *et al*., 2001; Brückner and Titgemeyer, 2002), and the phosphorylation state of the cell by HPr-Ser-P, which also contributes to this autoregulation (Saier *et al*., 1995; Darbon *et al*., 2002; Monedero *et al*., 2001). Therefore, depending on the phosphorylation state, the antiterminator SacT (transcriptional regulator) could activate the *sac*PA operon. Also, once the glycolytic phosphorylated intermediated increase (e.g., fructose-1,6-bisphosphate), the regulation mediated by CcpA occur (Sonenshein, 2007). Second, the activity of phosphosucrase (the enzyme that intracellularly hydrolyses sucrose) would be low, and therefore, the hydrolysis rate of the disaccharide is low. In addition, once the disaccharide is hydrolyzed, the glucose-6-phosphate enters the glycolytic pathway directly, while the entry of fructose to the glycolic

pathway could be a limiting step because it would need two previous phosphorylation steps to be incorporated as fructose-1,6-bisphosphate into the glycolysis. Furthermore, fructose-1,6-bisphosphate is an important metabolite that stimulates HPr kinase to phosphorylate Hpr and Crh, which interact with CcpA (a global regulator) regulating glycolytic flux (Sonenshein 2007; Deutscher *et al*., 2002). Third, the transport of sucrose is less active than glucose. On the other hand, as with glucose or fructose, with disaccharides, a yield higher than the theoretical was obtained (between 20 and 30% more, Table 4), indicating that the strain metabolizes carbon contained in the Luria medium to lactate.

B. subtilis can import arabinose under anaerobiosis by an ABC-type transporter protein. After entering the cell, arabinose is sequentially converted to ribulose, ribulose-5-phosphate, and xylulose-5-phosphate by the action of the arabinose isomerase ribulokinase and ribulose-5-phosphate epimerase, respectively. The regulation of the biosynthesis of these enzymes is transcriptional and is negatively controlled by the transcriptional factor *ara*R and by the presence of the inducer (arabinose). Once the inducer is present, AraR releases the operator sites in the promoter regions of araABDLMNPQ-abfA, *ara*E, and *ara*R operons (Rodionov *et al*., 2001). In our study, we observed low biomass formation with arabinose as compared to glucose and almost one order of magnitude lower volumetric rate of arabinose consumption compared to glucose. This may be due to two factors, one energetic and the other at the transcriptional level. The energy balance indicates that the catabolism of arabinose to pyruvate yields 0.67 mol ATP/mol arabinose (Nakano et al., 1998; Cruz-Ramos *et al*., 2000), while 2 mol ATP is obtained from the catabolism of 1 mol of glucose to pyruvate; hence the lower ATP yield with arabinose could hinder *B. subtilis* growth. On the other hand, it is possible that there was a low expression of genes that code for proteins that take up arabinose, as well as those genes encoding for the enzymes of the pentose phosphate pathway. Also, the metabolism of non-PTS carbohydrates can trigger PTS-mediated mechanisms to autoregulate the rate of sugar metabolism (Gunnewijk *et al*., 2001; Brückner and Titgemeyer, 2002). Besides, it has been reported that AraE, an unspecific transporter, is the most relevant transporter at a low concentration of arabinose (Krispin and Allmansberger, 1998). The expression of *ara*E depends on the negative transcriptional regulator AraR. Therefore, a low expression of *araE* or a low affinity of the AraE transporter for the substrate could cause a slow transport of arabinose and, therefore, a slow expression of the genes involved in arabinose catabolism (Sá-Nogueira and Ramos, 1997; Krispin and Allmansberger, 1998). Moreover, there is evidence that the metabolism of non-PTS carbohydrates influences the intracellular [PEP]/[pyruvate] ratio (Gunnewijk *et al*., 2001; Brückner and Titgemeyer, 2002), and this, in turn, influences PTS autoregulation mediated by CcpA and HPr-Ser-P. To our knowledge, there are no previous reports about the utilization of arabinose by *B. subtilis* under anaerobic conditions.

Conclusions

Besides glucose, *Bacillus subtilis* was found to consume fructose, cellobiose, sucrose, and arabinose under fermentation conditions and, depending on the genetic background, produced BDO and LA (*B. subtilis 168 trp*$^+$) or only LA (*B. subtilis ER382*, strain *168 trp*$^+$ Δ*als*S). Hexoses (glucose and fructose) were metabolized faster to LA by *B. subtilis ER382* than disaccharides and arabinose under non-aerated conditions. But neither strain could ferment glycerol or starch. In comparison to the progenitor strain, the deletion of *als*S eliminated the formation of butanediol, allowed to obtain LA as the only fermentation product and an increase in the biomass formed, which contributed to attain a higher LA volumetric productivity. Also, the volumetric LA productivity obtained from disaccharides was lower than hexoses metabolism, but the redox and energetic balance was maintained when hexoses and disaccharides were used as carbon sources, hence, future studies should focus on increasing the rate of sugar consumption to positively impact volumetric productivity. With the strain *ER382* and glucose, fructose, cellobiose or sucrose as carbon sources a LA yield higher than the theoretical was obtained, indicating that the strain metabolizes carbon contained in the Luria medium to lactate.

Declarations

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Authors' contribution OEC-R conceived the study, performed the experiments, made the data analysis, and designed the figures and drafted the manuscript. OEC-R, GG, and AM, designed the experiments, performed the data analysis, drafted, and edited the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1

Strains used in this study.

Strains and plasmids	Features	Source
E. coli XL1Blue	Tc^r, Nal^r. Transformable strain at high efficiencies and recombination deficient. Used for large-scale propagation of plasmids, filamentous phage, and phagemids.	Laboratory stock
E. coli XL1Blue / pJET::*lx*Spt*lx*	It contains the spectinomycin resistance gene flanked by the *lox*P sites cloned in plasmid pJET 1.2.	This study
E. coli XL1Blue /pTOPO*alsS*	Km^r, $Zeocin^r$. It contains the *als*S (*B. subtilis* acetolactate synthase) gene cloned in the pTOPO-blunt vector.	This study
E. coli XL1Blue / pTOPO*alsS*::*lx*Spt*lx*	Km^r, $Zeocin^r$. It contains the spectinomycin resistance gene from the plasmid pJET-Spt between the *als*S gene.	This study
B. subtilis 168 trp^+	Prototroph obtained from *B. subtilis* 168 trpC2 strain	Romero *et al*. [16]
B. subtilis (Δ*alsS*)	168 trp^+, *alsS::lxSptlx*	This study
B. subtilis ER382 (Δ*alsS*)	168 trp^+, $alsS^-$	This study

Table 2

Plasmids used in this study.

Plasmids	Features	Source
ploxSpec-Walt	Am^r, Sp^r. The expression vector has cloned the spectinomycin resistance gene flanked by *lox*P sites, which use the CRE recombinase.	Laboratory stock.
pCRM-Ts-Phleo	$Phleo^r$. Plasmid was used to cleave the resistance cassette by homologous recombination between the two *lox*P sites.	Cabrera *et al.* [25]
pJET1.2/blunt	Cloning vector of PCR products generated with DNA polymerases that generate blunt fragments. It contains a lethal gene (eco471R), which is disrupted by ligating a DNA insert at the cloning site.	FERMENTAS
pJET::*lx*Spt*lx*	Am^r. Contains cloned spectinomycin gene flanked by *lox*P sites.	This study
pTOPO*alsS*	Km^r. contains the *als*S gene from *B. subtilis*.	Romero *et al.* [21]
pTOPO*alsS::lxSptlx*	Km^r, $Zeocin^r$. Contains the gene for resistance to spectinomycin from the plasmid pJET::*lx*Spt*lx* between the *als*S gene of *B. subtilis*.	This study

Table 3

Oligonucleotides.

Primer	Name	Sequence (5' à 3')
1	SpecR_up	GGC CTA GGA TGC ATA TGG CGG CCG
2	SpecR-down	ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA TC
3	alsDinicio	GGC TGA GCA CTT AAA TGT TGC TTT C
4	alsS	GTG TCA CAC ATG TAA TTG GCA TTC C

Table 4

Fermentation parameters obtained for *B. subtilis* 168 trp^+ and *B. subtilis* ER382 (Δ*alsS*) cultivations under non-aerated conditions: 0.2 L fermenters at 37°C, pH 7.0 and 100 rpm, in Luria-Bertani medium amended with 10 g/L of glucose, fructose, cellobiose, sucrose or arabinose.

	X_{MAX} (g_X/L)		$Y_{X/S}$ (g_X/g_S)		LA (g_{LA}/L)		BDO (g_{BDO}/L)		$Y_{LA/S}$ (g_{LA}/g_S)		Q_{SUGAR} (g_{SUGAR}/L h)		Q_{LA} (g_{LA}/L h)	
Sugar / Strain	**168 trp^+**	**ER382**	**168 trp^+**	**ER382**	**168 trp^+**	**ER382**	**168 trp^+**	**ER382**	**168 trp^+**	**ER382**	**168 trp^+**	**ER382**	**168 trp^+**	**ER382**
Glucose	0.91	1.38	0.109	0.130	8.78	13.2	2.43	0	0.85	1.23	0.69	1.01	0.63	1.32
Fructose	ND	0.77	ND	0.087	ND	12.9	ND	0	ND	1.32	ND	0.78	ND	1.08
Cellobiose	0.58	0.67	0.058	0.070	12.2	12.5	1.15	0	1.25	1.36	0.22	0.23	0.27	0.31
Sucrose	0.63	0.84	0.064	0.091	12.7	12.2	1.67	0	1.44	1.31	0.17	0.19	0.24	0.24
Arabinose	0.54	0.84	0.097	0.099	6.80	8.4	1.63	0	1.27	0.98	0.08	0.13	0.10	0.13

The data are the average of two independent experiments. The parameters were calculated at the sugar depletion or end of the cultivations.

X_{MAX}: Maximum biomass.

$Y_{X/S}$: Biomass yield on consumed sugar.

LA: Lactic acid.

BDO: Butanediol.

$Y_{LA/S}$: Lactate yield on consumed sugar

Q_{LA}: Volumetric LA productivity.

Q_{SUGAR}: volumetric sugar consumption rate

ND: Not determined.

Figures

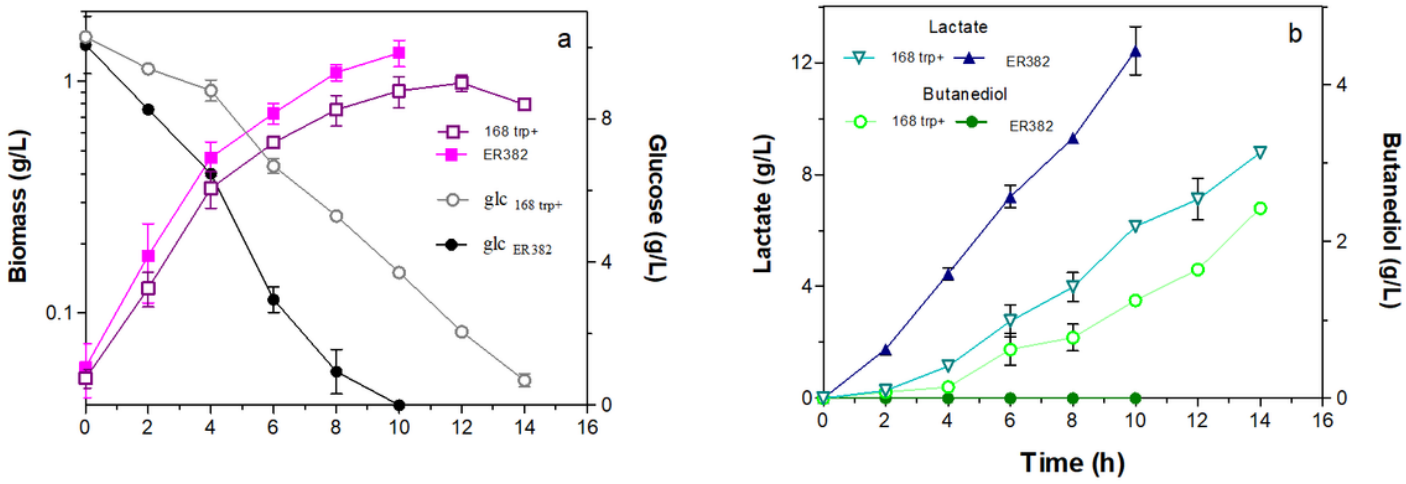

Figure 1

Fermentation of glucose (10 g/L) by *B. subtilis* in pH-controlled fermenters: strain 168 trp^+ (parent) (a) and strain ER382 (Δ*alsS*) (b).

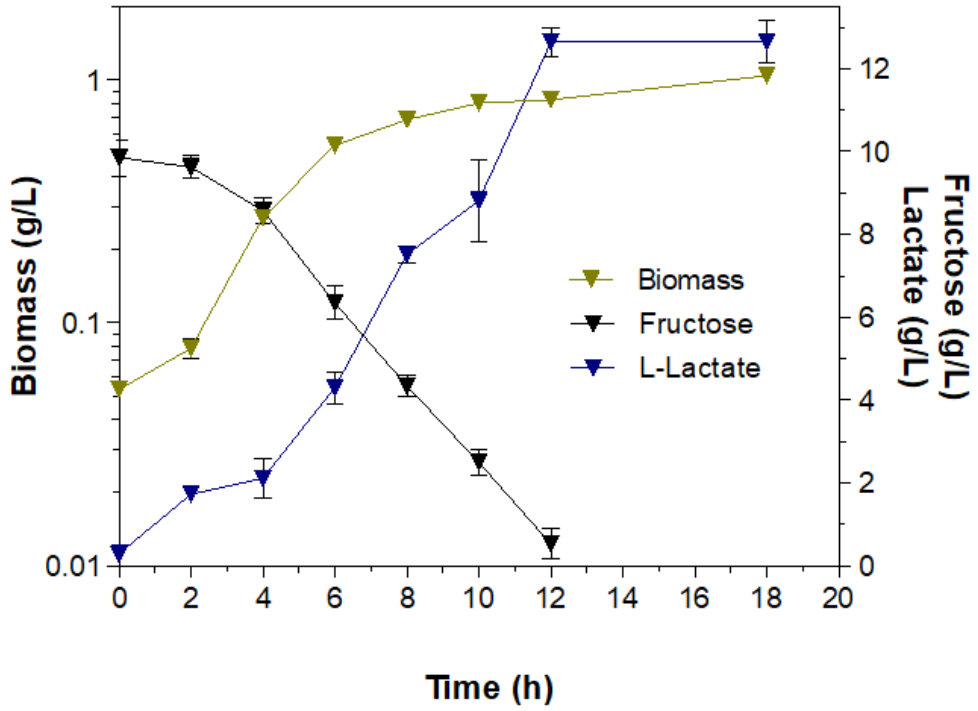

Figure 2

Fermentation of fructose (10 g/L) in pH-controlled fermenters by *B. subtilis* strain ER382 (Δ*alsS*).

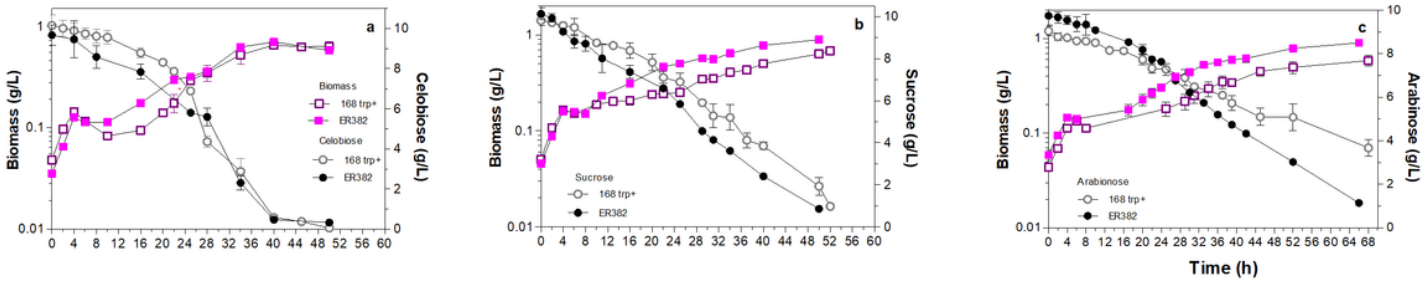

Figure 3

Fermentation of sugars (10 g/L), cell mass formation and residual sugars in pH-controlled fermenters by *B. subtilis* strains 168 trp$^+$ (parent) and ER382 (Δ*alsS*). Carbon sources: cellobiose (a), sucrose (b), and arabinose (c).

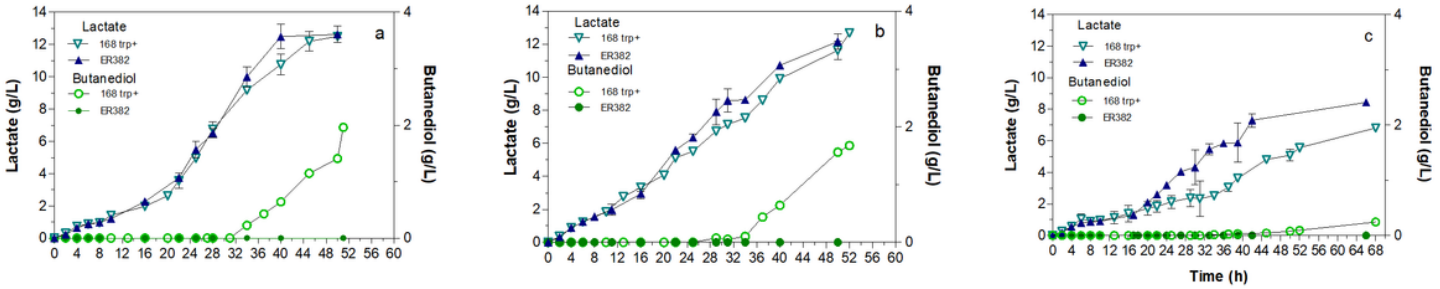

Figure 4

Fermentation products, lactate and 2,3-butanediol from *B. subtilis* strains 168 trp^+ (parent) and ER382 (Δ*als*S). Carbon sources: cellobiose (a), sucrose (b), and arabinose (c).