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Mechanisms of enhanced cellulosic bioethanol fermentation by co-cultivation of *Clostridium* and *Thermoanaerobacter* spp.

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ABSTRACT

Engineering microbial consortia capable of efficient ethanolic fermentation of cellulose is a strategy for the development of consolidated bioprocessing for bioethanol production. Co-cultures of cellulolytic *Clostridium thermocellum* with non-cellulolytic *Thermoanaerobacter* strains (X514 and 39E) significantly improved ethanol production by 194–440%. Strain X514 enhanced ethanolic fermentation much more effectively than strain 39E in co-cultivation, with ethanol production in X514 co-cultures at least 62% higher than that of 39E co-cultures. Comparative genome sequence analysis revealed that the higher ethanolic fermentation efficiency in strain X514 was associated with the presence of a complete vitamin B_{12} biosynthesis pathway, which is incomplete in strain 39E. The significance of the vitamin B_{12} *de novo* biosynthesis capacity was further supported by the observation of improved ethanol production in strain 39E by 203% following the addition of exogenous vitamin B_{12}. The vitamin B_{12} biosynthesis pathway provides a valuable biomarker for selecting metabolically robust strains for bioethanol production.

1. Introduction

Bioethanol has been established as one of the most promising renewable energy sources, particularly as a carbon–neutral liquid transport fuel (Agarwal, 2007; Carroll and Somerville, 2009). Currently, the majority of bioethanol is produced from grains such as corn, which is considered unsustainable with the growing competition between bioethanol feedstock supply versus food supply to utilize the same existing farmland and water resources (Chakravortya et al., 2008; Simpson et al., 2008). Compared to grain-derived bioethanol, cellulosic bioethanol is a more economically feasible alternative given the abundance of cellulosic biomass.

However, the recalcitrance of cellulosic feedstock to biotransformation has hindered the development of cost-effective processes for cellulosic bioethanol, which typically involve multiple steps of physicochemical pretreatment, enzymatic hydrolysis, and ethanolic fermentation. To minimize the processing steps and enhance efficiency, the consolidated bio-processing (CBP) concept has been proposed to combine all biochemical steps involved in cellulosic bioethanol production into a single bioconversion process (Lynd et al., 2002), representing a potential technological advance that could lead to the largest reduction in processing costs for cellulosic bioethanol production (Lynd et al., 2008). One strategy to developing CBP is the genetic engineering of microbial cultures capable of both efficient cellulose utilization and carbohydrate fermentation (Lynd et al., 2005), which is currently limited by the availability of effective genetic tools. An alternative is the development of microbial consortia consisting of metabolically complementary microbial populations capable of carrying out the suite of bioconversion steps required for bioethanol production from cellulose. The use of mixed culture microbial consortia for bioconversion, however, poses a challenge to process control, requiring understanding of the interactions between microbial populations, particularly those with coupled metabolic functions.

One such interaction is the relationship between cellulolytic and ethanologenic fermentative bacteria, which form a critical bioconversion tandem for cellulosic bioethanol production. Capable of thermophilic cellulolysis, *Clostridium thermocellum* has been the model organism for CBP development (Demain et al., 2005); but its application has been limited by the low ethanol yields in carbohydrate fermentation by this bacterium (Beguin and Aubert, 1994; Lynd, 1989). Subsequently, it was proposed that co-cultivation of *C. thermocellum* with another fermentative bacterium efficient in ethanolic carbohydrate fermentation would improve ethanol production, which was first demonstrated in an early study where

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the co-cultures of *C. thermocellum* and *Thermoanaerobacter pseudethanolicus* 39E significantly enhanced ethanol yields from cellulose fermentation (Ng et al., 1981). A recent study further explored co-cultures consisting of *C. thermocellum* and various *Thermoanaerobacter* strains as the ethanolic fermentative partner, revealing considerable variations in the ability of the co-cultures to enhance ethanol production, with the highest ethanol yield observed in the co-culture of *C. thermocellum* and *Thermoanaerobacter* sp. strain X514 (Fang et al., 2008).

Therefore, the objective of this study was to identify characteristics contributing to the disparities in the ability of different *Thermoanaerobacter* strains to enhance cellulosic bioethanol production in co-culture with *C. thermocellum*. Two *Thermoanaerobacter* strains, 39E and X514, were compared with physiological and genomic analysis. Our results show that the presence of the complete pathway for vitamin B_{12} biosynthesis is a key determinant of ethanolic fermentation efficiency in co-cultivation.

2. Methods

2.1. Cellulosic substrates

To ensure consistency in the testing of cellulose fermentation, only commercially available cellulosic substrates from the same delivery were used in this study. Two powdered cellulosic substrates, Avicel® and Solka Floc®, were tested in this study. Avicel PH-101 was purchased from Sigma–Aldrich Co., St. Louis, Missouri. Solka Floc 200 was acquired from International Fiber Corporation, North Tonawanda, New York.

2.2. Microorganisms

Clostridium thermocellum (ATCC 35609) and *Thermoanaerobacter pseudethanolicus* strain 39E (ATCC 33323) were obtained from the American Type Culture Collection (Manassas, Virginia). *Thermoanaerobacter* sp. strain X514 was originally isolated from the deep subsurface in the Piceance Basin, Colorado (Roh et al., 2002) and is maintained in our laboratory culture collection. Strain X514 has been deposited at the American Type Culture Collection (ATCC BAA-938). *C. thermocellum* is cellulolytic (McBee, 1954); but strains X514 and 39E are non-cellulolytic and strictly fermentative (Roh et al., 2002; Zeikus et al., 1980).

2.3. Medium formulation and preparation

A defined anaerobic medium was used throughout this study according to a previously described formula (He et al., 2009). The medium contained the following (per liter): 10.0 g NaCl, 0.5 g $MgCl_2{\cdot}6H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.015 g $CaCl_2{\cdot}2H_2O$, 1 mL trace element solution, 1 mL selenium-tungsten solution, 10 mL vitamin solution, 2.52 g $NaHCO_3$, and 0.05 mg resazurin. The trace element solution contained the following (per liter): 1.5 g $FeCl_2{\cdot}4H_2O$, 0.19 g $CoCl_2{\cdot}6H_2O$, 0.1 g $MnCl_2{\cdot}4H_2O$, 70 mg $ZnCl_2$, 6 mg H_3BO_3, 36 mg $Na_2MoO_4{\cdot}2H_2O$, 24 mg $NiCl_2{\cdot}6H_2O$, and 2 mg $CuCl_2{\cdot}2H_2O$. The selenium-tungsten solution contained 6 mg $Na_2SeO_3{\cdot}5H_2O$ per liter, 8 mg $Na_2WO_4{\cdot}2H_2O$ per liter, and 0.54 g of NaOH per liter. The vitamin solution contained the following (per liter): 20 mg biotin, 20 mg folic acid, 100 mg pyridoxine hydrochloride, 50 mg riboflavin, 50 mg thiamine, 50 mg nicotinic acid, 50 mg pantothenic acid, 1 mg vitamin B_{12}, 50 mg *p*-aminobenzoic acid, and 50 mg thioctic acid.

The pH of the medium was adjusted to 7.2 ± 0.1 by purging with an oxygen-free nitrogen/CO_2 gas mix. Anaerobic condition of the medium was maintained by the addition of sulfide (0.048 g $Na_2S{\cdot}9H_2O$) and cysteine (0.031 g L-cysteine) as reductants as previously described (He and Sanford, 2002). The medium was sterilized by autoclaving following dispensing into sealed glass containers. The vitamin solution was added from sterile anaerobic stock solutions after autoclaving.

2.4. Growth conditions

Stock cultures of all bacterial strains were stored in 15% glycerol at −80 °C. Inocula were obtained by sub-culturing aliquots of the frozen stocks in 60-mL serum bottles with 30 ml of boiled degassed medium closed with butyl rubber stoppers and aluminum seals. Cultures were incubated in the dark at 60 °C without constant agitation. For routine cultivation, 1% (w/v) of cellobiose (for the cellulolytic culture *C. thermocellum*) or glucose (for the non-cellulolytic 39E and X514) was added as the only fermentation substrate in defined medium. Active cultures were maintained by transferring a 1% inoculum to fresh medium after fermentation was completed and growth had stopped. Strict anaerobic techniques were used throughout all experimental manipulations. Sterile syringes and needles, used for substrate addition and sampling, were flushed with N_2 prior to use.

2.5. Cellulose fermentation

Cellulose fermentation experiments were initiated by a 1% (v/v) inoculum of log-phase cultures ($OD_{600} \sim 0.5$) grown on cellobiose or glucose. Mono-cultures were inoculated with *C. thermocellum* only, referred to as CT mono-cultures. Co-cultures were inoculated with strain X514 or 39E in addition to *C. thermocellum*, referred to as CT-X514 or CT-39E co-cultures, respectively. In fermentation experiments to determine the role of non-cellulolytic *Thermoanaerobacter* strains in cellulose fermentation, co-cultures were established stepwise by first initiating mono-cultures of *C. thermocellum* followed by the addition of strain 39E or X514 when soluble sugar had accumulated. When needed, yeast extract 0.6% (w/v) was added to the defined medium during medium preparation. In fermentation experiments where exogenous vitamin B_{12} was added to the defined medium, an alternative vitamin solution was made by omitting vitamin B_{12} from the formula and used for the preparation of the defined medium. Subsequently, varying amounts of vitamin B_{12} was added before inoculation. All fermentation experiments were performed in triplicates.

2.6. Analytical procedures

To monitor the production of fermentation end products, samples (1 mL) from the culture broth were taken periodically via degassed sterile syringes and filtered through Millipore GSWP 0.20-μm filters prior to analysis. Ethanol was determined with a 6890 chromatography apparatus (Agilent Technologies, Santa Clara, California) following a previously described protocol (He et al., 2009). Soluble reducing sugar in the culture broth was quantified with the phenol–sulfuric acid method as previously described (Daniels et al., 1994). Cellulose utilization was determined by the decrease in cellulose concentration as previously described (Updegraf, 1969). Briefly, 1-mL samples of fermentation broth were harvested. Solids were collected as pellets by centrifugation at 5000*g* for 15 min. The pellets were suspended in distilled water and heated at 100 °C for 30 min to remove cell mass by cell lysis. The remaining cellulose fraction was washed with distilled water for two times through centrifugation at 5000*g* for 15 min, and then hydrolyzed into soluble sugars with 65% H_2SO_4. The soluble sugars derived from cellulose were assayed with the phenol–sulfuric acid method (Daniels et al., 1994).

2.7. Statistical analysis

Differences in ethanol yields between cultures were analyzed using the one-way analysis of variance (ANOVA). If significant differences were indicated by a probability value less than 0.05 in ANOVA analysis, post hoc comparisons were performed using Tukey's test to further identify the pairs of cultures with significant difference as indicated by a probability value less than 0.05. Statistical analyses were performed with JMP 7.0.1 for Windows (SAS Institute Inc., Cary, North Carolina) as described previously (He et al., 2009).

2.8. Genome sequence analysis

The sequences and annotations of the complete *T. pseudethanolicus* 39E and *Thermoanaerobacter* sp. X514 genomes are available at GenBank/EMBL/DDBJ under accession numbers CP000924 and CP000923, respectively. Both genome sequences are also available at the JGI-IMG website (http://img.jgi.doe.gov/pub/main.cgi). Genome sequence analysis, including metabolic pathway analysis and ortholog detection, was conducted primarily using online tools at the Joint Genome Institute Integrated Microbial Genomes database (http://img.jgi.doe.gov/pub/main.cgi) (Markowitz et al., 2006) or manually using ad hoc Perl scripts.

3. Results and discussion

3.1. Performance of ethanolic fermentation of cellulose in co-cultures

To evaluate the efficiency of cellulose utilization and ethanol yields in co-cultivation, ethanol production was monitored in the *C. thermocellum* (CT) mono-cultures and co-cultures of *C. thermocellum* with strains 39E (CT-39E) or X514 (CT-X514). The CT mono-cultures exhibited ethanol yields (<13 mM) much lower than those of the two co-cultures tested in this study with 1% (w/v) of cellulose in defined medium (Fig. 1), consistent with previous reports on the low ethanolic fermentation efficiency of *C. thermocellum* (Lynd, 1989). The highest ethanol yields (>60 mM) were found in the CT-X514 co-cultures while much lower values (~37 mM) were observed in the CT-39E co-cultures, suggesting strain X514 as a more efficient partner strain with *C. thermocellum* for cellulose fermentation. Nonetheless, ethanol production from cellulose was greatly improved by the co-cultivation of *C. thermocellum* with either *Thermoanaerobacter* strain as the fermentative partner.

Despite the considerable variations in ethanol yields observed between different cultures, the two different cellulosic substrates, i.e. Solka Floc and Avicel, resulted in nearly identical ethanol yields in the same cultures (Fig. 1), indicating that substrate characteristics did not contribute to the differences in ethanolic fermentation between the CT-39E and CT-X514 co-cultures.

3.2. Linkage between soluble sugar accumulation and ethanol production

To ascertain the metabolic characteristics contributing to the superior capacity of strain X514 as compared to strain 39E in enhancing ethanolic fermentation, a closer examination of the cellulose fermentation process was performed. Corresponding to the greater ethanol production in the CT-X514 co-cultures than that in the CT-39E co-cultures (Fig. 2A), soluble sugar level was higher in the CT-39E co-cultures than that in the CT-X514 co-cultures (Fig. 2B). Since cellulose utilization in both co-cultures was nearly complete with greater than 90% of the added cellulose (1.0%) converted into soluble sugar (Fig. 2B), the accumulative soluble sugar released from cellulose decomposition was thus comparable between the CT-X514 and CT-39E co-cultures. Therefore, the higher level of soluble sugar accumulation and lower ethanol yield in the CT-39E co-cultures, as compared to those in the CT-X514 co-

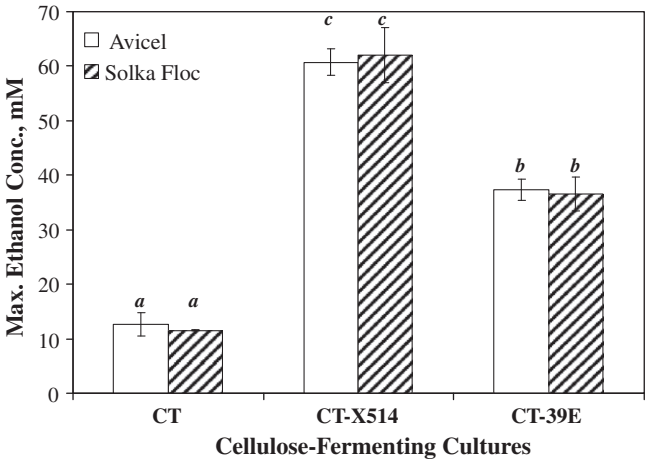

Fig. 1. Maximum ethanol yields in *C. thermocellum* (CT) and its co-cultures with *Thermoanaerobacter* strains X514 and 39E in batch cellulose fermentation in defined medium. The concentrations of the two cellulosic substrates tested, Avicel and Solka Floc, were 1% (w/v). Data were means of triplicate cultures with the error bars showing the standard deviations. Ethanol yields are not significantly different from each other if labeled with the same lowercase letters on top of the data columns (ANOVA, Tukey's test, $p < 0.05$).

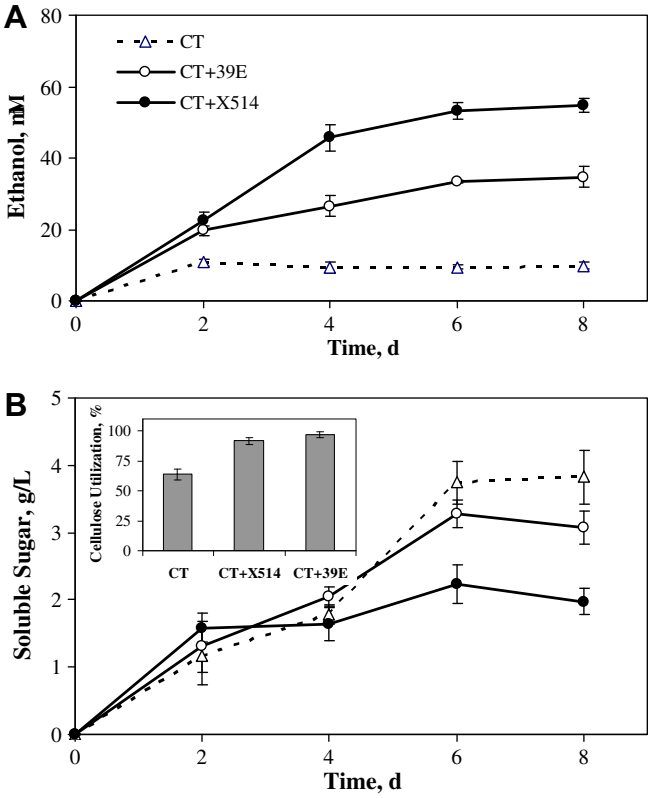

Fig. 2. Time course conversion of Solka Floc (1%) as the cellulosic substrate in batch fermentation by *C. thermocellum* (CT) and its co-cultures with *Thermoanaerobacter* strains X514 and 39E in defined medium. (A) Production of ethanol; and (B) accumulation of soluble reducing sugar in the culture broth with the inset showing the percentage utilization of Solka Floc. Data are means of triplicate cultures with the error bars showing the standard deviations.

cultures, were likely the result of less efficient sugar utilization by strain 39E than X514. It is unlikely that the lower ethanol yields by 39E could be attributed to a more severe inhibitory effect by ethanol, because no inhibition in ethanolic fermentation was observed at ethanol levels much higher than those in this study (Ng et al., 1981).

Similarly, the low ethanol yields associated with the CT mono-cultures coincided with the highest level of soluble sugar accumulation in the culture broth, even though the CT mono-cultures utilized only 64 ± 4% of the added cellulose, which was much lower than those achieved in the co-cultures (>90%) (Fig. 2). It is thus plausible that the lower ethanol yields in the CT mono-cultures as compared to those in the co-cultures could be attributed to the lack of efficient carbohydrate fermentation capacity in *C. thermocellum*, which resulted in the accumulation of carbohydrates. Since soluble carbohydrates such as cellobiose and glucose have been shown to inhibit the cellulolytic activity of *C. thermocellum* (Johnson et al., 1982), inefficient fermentation and subsequent accumulation of soluble carbohydrates were likely the cause of the relatively poor cellulose utilization and low ethanol production in the CT mono-culture (Fig. 2).

3.3. Role of non-cellulolytic fermentative partner cultures in enhancing cellulose fermentation

To validate that the ability of the non-cellulolytic *Thermoanaerobacter* strains to enhance ethanol production from cellulose in co-cultivation was attributable to the efficient utilization and removal of soluble sugar librated from cellulolysis, co-cultures were established stepwise by first initiating CT mono-cultures followed by the addition of strain 39E or X514 when soluble sugar had accumulated. Indeed, the addition of the fermentative *Thermoanaerobacter* strains improved ethanol production, with the CT-X514 co-cultures exhibiting the highest ethanol yield (Fig. 3A). Accordingly, the CT mono-cultures accumulated the highest level of soluble sugar in parallel with the lowest ethanol yields (Fig. 3B), supporting the importance of sugar accumulation in cellulose fermentation. It is evident that the addition of strain X514 to the CT mono-cultures rapidly lowered sugar concentration, which is likely the result of enhanced sugar utilization by strain X514 (Fig. 3B). The reduction in soluble sugar concentration following the addition of X514 was also accompanied with the simultaneous increase in ethanol production (Fig. 3A), illustrating the role of strain X514 in efficient carbohydrate utilization and ethanol production, which was deficient in *C. thermocellum*.

In contrast, the addition of strain 39E resulted in only limited reduction in soluble sugar accumulation as compared to the CT mono-cultures (Fig. 3B), which was in line with the modest improvement in ethanol yields in the CT-39E co-cultures (Fig. 3A). These results validated the significance of efficient carbohydrate utilization in cellulose fermentation and the importance of the fermentative *Thermoanaerobacter* strains in enhancing cellulosic ethanol production.

3.4. Stimulatory effect of yeast extract

The addition of yeast extract has been shown to significantly enhance ethanolic fermentation (He et al., 2009; Sato et al., 1992). However, the responses of co-cultures with coupled metabolic functions to yeast extract addition may differ from those of mono-cultures due to metabolic constraints imposed upon by the partner cultures. Indeed, the addition of 0.6% yeast extract (w/v) considerably enhanced ethanol yields in both the CT-X514 and CT-39E co-cultures fermenting 1% cellulose, by 78% and 206%, respectively (Fig. 4A). Evidently, the greater increase in ethanol yields in the CT-39E co-cultures indicates that strain 39E benefited

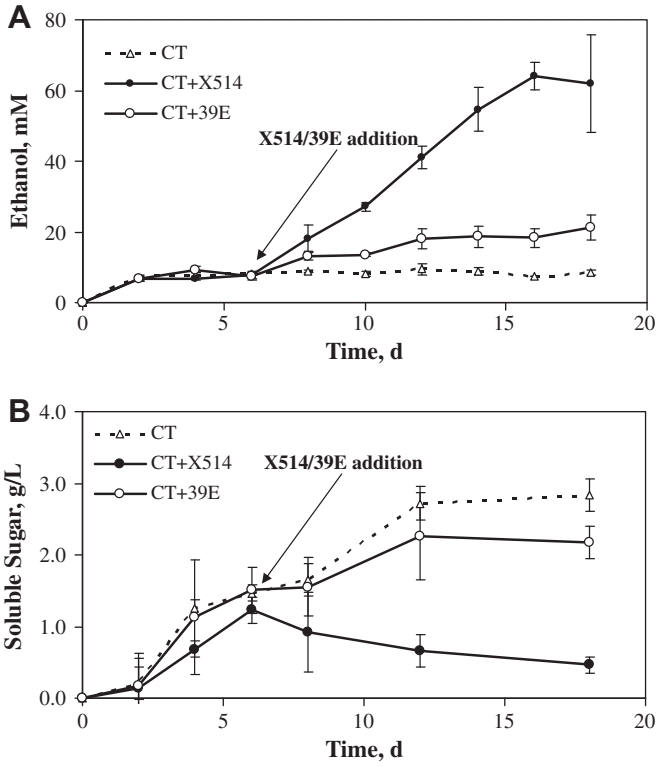

Fig. 3. Enhancement of ethanol fermentation with the addition of strain X514 or 39E following six days of cellulose fermentation by *C. thermocellum* (CT) mono-cultures in defined medium. (A) Production of ethanol; and (B) accumulation of soluble reducing sugar in the culture broth. The cellulosic substrate was Avicel with an initial concentration of 1% (w/v). Data are means of triplicate cultures with the error bars showing the standard deviations.

much more from the presence of yeast extract than strain X514 did. As a result, ethanol yields in CT-39E co-cultures, which were consistently lower than those in the CT-X514 co-cultures in defined medium without yeast extract (Fig. 1), reached the same level of ethanol production as that in the CT-X514 co-cultures following addition of yeast extract (Fig. 4A). Thus the presence of yeast extract appeared to have compensated for the deficiency of strain 39E in ethanol production in defined medium, which is likely due to the enhanced sugar utilization capability of strain 39E with the presence of yeast extract as evidenced by the substantial reduction in soluble sugar accumulation (Fig. 4A). These results again confirmed the importance of the efficiency of carbohydrate utilization in enhancing cellulose fermentation by co-cultivation. Surprisingly, the CT-X514 co-cultures, which exhibited the best ethanol yields and cellulose utilization in defined medium (Fig. 2), did not benefit from yeast extract supplementation at the same extent as the CT-39E co-cultures did, suggesting the presence of potential differences in the metabolic characteristics between strains X514 and 39E.

The stimulatory effect of yeast extract on both co-cultures was further studied at greater initial cellulose concentrations. It is found that the addition of 0.6% yeast extract considerably improved ethanol production in both co-cultures at all three cellulose concentrations tested, i.e. 1%, 2%, and 5%, as compared to the ethanol yields in co-cultures grown in defined medium without yeast extract (Fig. 4B). However, the two co-cultures responded differently to the increases in the concentration of the cellulose substrate. More specifically, the ethanol yields in the CT-X514 co-cultures increased steadily from 102 ± 6 to 164 ± 55 mM as the concentration of the cellulose substrate was raised from 1% to 5%. In contrast, the ethanol yields in the CT-39E co-cultures

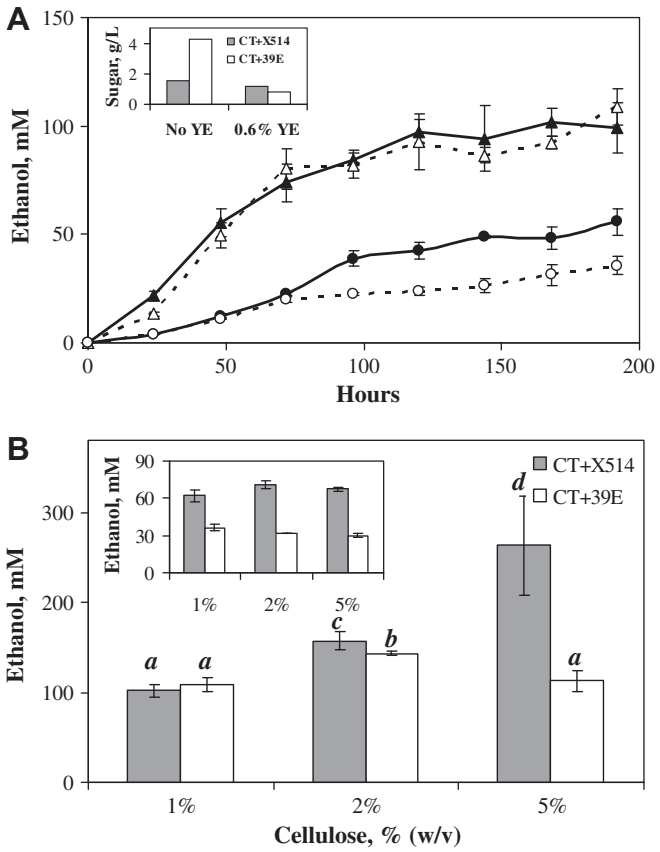

Fig. 4. Impact of yeast extract on ethanolic fermentation of Solka Floc by *C. thermocellum* (CT) and its co-cultures with *Thermoanaerobacter* strains X514 and 39E. (A) Time course ethanol production from fermentation of 1% Solka Floc in co-cultures with (triangles) or without (circles) yeast extract supplement. Solid symbols: CT-X514; and open symbols: CT-39E. The inset shows the formation of soluble reducing sugar in the fermentation of 1% Solka Floc in co-cultures supplemented with or without yeast extract (YE). (B) Ethanol yields from fermentation of Solka Floc at various initial concentrations in co-cultures supplemented with yeast extract. The inset shows the ethanol yields by co-cultures fermenting cellulose at various initial concentrations in defined medium without yeast extract. The concentration of yeast extract was 0.6% (w/v) when added as a supplement. Data are means of triplicate cultures with the error bars showing the standard deviations. Ethanol yields are not significantly different from each other if labeled with the same lowercase letters on top of the data columns (ANOVA, Tukey's test, $p < 0.05$).

showed an initial increase from 109 ± 8 mM (1% cellulose) to 143 ± 3 mM (2% cellulose). However, the stimulatory effect diminished as ethanol yields declined to 113 ± 12 mM when cellulose level was raised further to 5% cellulose. Since yeast extract is considered to be rich in growth-stimulating factors (Stokes et al., 1944), the reduction in the stimulatory effect of yeast extract at higher substrate levels suggests the possibility that a growth factor might have become limiting in the CT-39E co-cultures. In contrast, the CT-X514 co-cultures did not appear to be limited by a growth factor with continued improvement in ethanolic fermentation at higher substrate levels. These results further highlighted the potential differences in metabolic functions between strains X514 and 39E, likely related to the ingredients of yeast extract, such as potential growth factors.

3.5. Characteristics of the vitamin B_{12} biosynthesis pathway

In order to identify metabolic characteristics responsible for the divergent responses to yeast extract addition in co-cultures of CT-X514 and CT-39E, a comparative analysis was conducted on the recently available whole genome sequences of strains X514 and 39E (Hemme et al., 2010). A contiguous cluster of 21 genes encoding putative B_{12}-dependent enzymes were identified in the two *Thermoanaerobacter* genomes, including genes in the biosynthesis of tetrapyrole, corrin ring, and vitamin B_{12} (Fig. 5). Strain X514 was found to encode a complete B_{12} biosynthesis operon whereas no genes were found in the genome of strain 39E to encode the anaerobic corrin ring biosynthesis portion of the pathway (*cbiCDET* and *cbiFGHJ-hemA*) (Fig. 5).

Thus for strain 39E to actively perform ethanolic fermentation, a sustained supply of excess vitamin B_{12} would be necessary, which might not be the case for batch cultivation in defined medium. In contrast, strain X514, having a complete vitamin B_{12} biosynthetic pathway, would not require the presence of exogenous vitamin B_{12} to perform ethanolic fermentation, which is consistent with the greater ethanol production in defined medium by strain X514 than that by strain 39E in co-cultivation with *C. thermocellum* (Fig. 1). The potential of vitamin B_{12} as a limiting growth factor for strain 39E but not strain X514 could also explain the differences in the responses of the co-cultures to the addition of yeast extract as a rich source of growth factors. Since strain 39E requires exogenous vitamin B_{12}, the addition of vitamin B_{12}-containing yeast extract would stimulate the activity of strain 39E, and hence the significant increase of 206% in ethanol yields in the CT-39E co-cultures following the amendment of 0.6% yeast extract (Fig. 4A). Accordingly, since strain X514 does not require vitamin B_{12} from exogenous sources, the stimulatory effect of yeast extract addition would be less significant, resulting in an increase of 78% in ethanol yields in the CT-X514 co-cultures. These results suggest that the capacity for vitamin B_{12} biosynthesis could be an important determinant of ethanolic fermentation efficiency by *Thermoanaerobacter* strains in co-cultivation with *C. thermocellum*.

3.6. Validation of the roles of exogenous vitamin B_{12}

Given the potential importance of vitamin B_{12} suggested by genomic analysis, the response of ethanolic fermentation to vitamin B_{12} addition was monitored in defined medium. When the culture medium was devoid of vitamin B_{12}, the ethanol yields in the CT-39E co-cultures were only 32% of those in the CT-X514 co-cultures (Fig. 6), supporting the results from genomics analysis that the lack of the complete vitamin B_{12} biosynthesis pathway in strain 39E would impair fermentation efficiency when sufficient exogenous vitamin B_{12} was not available. Ethanolic fermentation in the CT-39E co-cultures steadily improved with increasing concentrations of exogenous vitamin B_{12}, ultimately approaching the ethanol yields in the CT-X514 co-cultures when vitamin B_{12} reached 30 μg/L (Fig. 6), confirming the importance of vitamin B_{12} in ethanolic fermentation. In contrast, the addition of exogenous vitamin B_{12} had no impact on the CT-X514 co-cultures as ethanol yields remained unchanged over the concentration range of vitamin B_{12} tested in this study (Fig. 6). These results suggest that the capacity for *de novo* vitamin B_{12} biosynthesis is an important metabolic feature conferring strain X514 high fermentation efficiency, which could be used as a biomarker for efficient ethanolic fermentation.

Recently, a rapidly increasing number of microbial cultures capable of ethanolic fermentation have become available for applications in the production of cellulosic bioethanol, particularly in consolidated bioprocessing (CBP). Thus, the significance of the vitamin B_{12} *de novo* biosynthesis in ethanolic fermentation identified in this study needs to be validated in other ethanologenic bacteria and could potentially provide a valuable biomarker for the selection of metabolically robust strains suitable for cellulosic

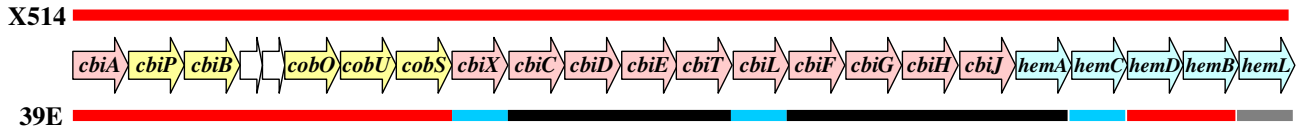

Fig. 5. Genomic arrangements of the vitamin B_{12} biosynthesis pathway in *Thermoanaerobacter* strains X514 and 39E. Operon structure of protein-encoding genes (arrows) represents the three stages of the vitamin B_{12} biosynthesis pathway: tetrapyrole biosynthesis (turquoise); corrin ring biosynthesis (pink); and final vitamin B_{12} biosynthesis (yellow). Genes encoding hypothetical proteins are white. The colors of the line segments indicate the standing of each vitamin B_{12} biosynthesis gene in strain X514 or 39E: present (red), present but degraded or truncated (blue), not identified in the genome (black), or presumed gene fusion (grey, in this case representing a bifunctional siroheme synthase/glutamate-1-semialdehyde aminotransferase).

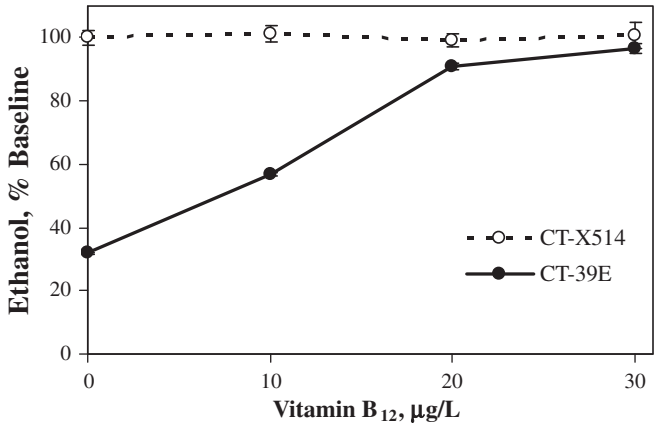

Fig. 6. Impact of vitamin B_{12} addition on ethanol production in cellulose fermentation by co-culture of *C. thermocellum* (CT) and *Thermoanaerobacter* strains X514 or 39E. Data shown are ethanol yields as a percentage of the baseline value, which is the ethanol yield by the CT-X514 co-culture in defined medium with no B_{12} addition. The concentrations of the cellulosic substrate Avicel was 1% (w/v). Data are means of triplicate cultures with the error bars showing the standard deviations.

bioethanol production as well as the development of efficient process control through optimized nutrient supply.

4. Conclusions

Cellulolytic bacteria such as *C. thermocellum* are highly efficient in cellulose degradation; but not so in ethanolic fermentation. Co-cultivation of *C. thermocellum* with non-cellulolytic *Thermoanaerobacter* strains (X514 and 39E) considerably improved ethanol production from cellulose. Strain X514 enhanced ethanolic fermentation much more effectively than strain 39E, which was attributed to the vitamin B_{12} biosynthesis capacity of strain X514. Thus, vitamin B_{12} and its metabolic intermediates could be used as valuable nutrient supplements to optimize the fermentative production of bioethanol. Additionally, the vitamin B_{12} *de novo* biosynthesis pathway provides a potential biomarker for the selection of strains with high ethanolic fermentation potential for the development of more efficient cellulosic bioethanolic production processes. Future efforts are needed to survey the distribution of the vitamin B_{12} biosynthesis pathway in other ethanologenic strains and further validate the importance of this pathway as a key determinant of ethanolic fermentation efficiency.

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