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A novel assay for rapid in vivo determination of phenotypic stability of recombinant ethanol-producing microorganisms

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ABSTRACT

A rapid empirical assay is presented for assessing the phenotypic stability of continuous cultures of recombinant bacteria containing transposed *pdc* and *adh* genes for ethanol production. The method measures spectrophotometrically the rate of colour formation when cells oxidize added ethanol to acetaldehyde in the presence of Schiff's reagent. During chemostat cultures of the recombinant ethanologen *Escherichia coli* KO11 on 20 g/l glucose, assay activities were stable and high at *ca* 8×10^{-4} $\Delta OD_{540}/(s.OD_{550})$, reflecting the high, stable ethanol yield (*ca* 95%). On 20 g/l and 50 g/l xylose, ethanol yields declined rapidly to about 60% and this was closely mirrored by the assay activities which fell to *ca* 1.5 $\Delta OD_{540}/(s.OD_{550})$, only slightly higher than those measured for the parent strain. Typically taking only about an hour to perform, the assay provides a faster means of gauging the phenotypic stability of ethanol production than is possible by conventional methods.

1. Introduction

The production of ethanol from fermentation of renewable lignocellulosic biomass such as wood has the potential to augment world liquid fuel supplies and reduce net CO_2 generation without competing with food production (Gomez et al., 2008). However for the process to be economic, the entire carbohydrate content of the substrate needs to be fermentable to ethanol. This includes the hemicellulose sugars which, although accounting for as much as 40% of the total carbohydrate, are mainly unfermentable by traditional yeast strains (Galbe et al., 2007). Recently several different approaches have been adopted to construct recombinant microorganisms that can successfully ferment these sugars (Jarboe et al., 2007; Jeffries, 2006). This work focuses on one such approach, which has entailed inserting the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) genes from the highly ethanologenic bacterium *Zymomonas mobilis* into bacteria that do not normally produce ethanol at high-yield but which can take up the hemicellulose sugars. When expressed at high levels, the transposed genes divert pyruvate metabolism from acid production to high-yield ethanol production (Doran Peterson and Ingram, 2008). This technique has been successfully implemented with several different bacteria, including *Escherichia coli* (Dien et al., 2000; Ingram et al., 1987), *Klesbiella oxytoca* (Ohta et al., 1991) and *Erwinia* strains (Beall and Ingram, 1993).

To increase productivity in fuel ethanol production, continuous fermentation is widely employed in large-scale starch-to-ethanol plants. In a continuous lignocellulose-to-ethanol process, the long-term stability of the recombinant strain is a matter for concern, particularly if there is a potential for the transposed ethanol-producing genes to be lost or for their expression to diminish. This raises the need for laboratory techniques capable of rapidly assessing the stability of a culture during fermentation. Existing attempts to evaluate stability in recombinant ethanol fermentations have either been based on plate-counting to determine the extent of retention of antibiotic markers (Dien et al., 2000; Ohta et al., 1991; Yomano et al., 1998) or have relied on daily measurements of the fermenter ethanol concentration as an indicator of phenotypic stability (Dien et al., 2000; Dumsday et al., 1999; Martin et al., 2006). Both methods are slow, due to the long times required to incubate plate cultures and the usual need to monitor the ethanol concentration for several days before any trend to decreasing ethanologenicity can be definitively identified.

The aim of this work was to develop a rapid empirical method for assessing the phenotypic stability of continuous cultures of recombinant bacteria containing transposed *pdc* and *adh* genes for ethanol production. Ideally such a procedure would be able to predict any impending loss of ethanol-producing ability without the need for plate incubations, colony counting or protracted monitoring of the fermenter ethanol concentration and would be performable in as little as 1 h of assay time.

The method proposed (termed here the RBS or rosanilin bisulphite assay) is a quantitative spectrophotometric assay based on the chemistry of the aldehyde indicator plate technique used qual-

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itatively by Ingram and colleagues to detect high ethanol-producing colonies of their ethanologenic *E. coli* strains (Conway et al., 1987; Ingram et al., 1990; Ingram and Conway, 1992; Ingram et al., 1987; Keshav et al., 1990; Ohta et al., 1991). The method provides an empirical measure of the overall alcohol dehydrogenase activity of liquid culture samples, based on the rate of red colour formation when added ethanol is oxidized to acetaldehyde in the presence of Schiff's reagent. The procedure was tested using the recombinant *E. coli* strain KO11 developed by Ingram and colleagues for commercial ethanol production from lignocellulose (Ohta et al., 1991). This strain is well suited to this purpose since, while producing high ethanol yields in batch culture (Dien et al., 2003; Doran Peterson and Ingram, 2008; Hahn-Hägerdal et al., 1994; Lima et al., 2002; Rao et al., 2007), it shows progressively declining yields in continuous culture when cultivated on sugars other than glucose (Dumsday et al., 1997, 1999; Zhou et al., 2008), presumably due to reduced expression or loss of the transposed ethanol-producing genes (Dumsday et al., 1999).

2. Methods

2.1. Strain

Escherichia coli strains KO11 and ATCC 11303 were obtained from Professor L.O. Ingram (University of Florida, Gainesville, FL, USA) and the American Type Culture Collection (Rockland, MD), respectively, and were maintained lyophilised in ampoules. A fresh ampoule was used to start a new culture for each experiment.

2.2. Media

The base medium was modified Luria broth which contained: tryptone (Oxoid, Australia), 10 g/l; yeast extract (Oxoid, Australia), 5 g/l; and NaCl, 5 g/l. The carbon source was either D-glucose or D-xylose (Sigma, Australia) at 20 g/l or 50 g/l as described in the text. Feed medium for the continuous fermentation experiments was filter-sterilised. All other media were sterilised by autoclaving at 121 °C for 20 min. No antibiotics were used in the fermentation experiments.

2.3. Inoculum preparation

Inocula were prepared by reviving, growing and harvesting cells stored in freeze-dried ampoules as described previously (Zhou et al., 2008).

2.4. Continuous cultivations

Experiments with 20 g/l glucose or xylose feed were performed in a New Brunswick model 19 bioreactor (New Brunswick Scientific, New Brunswick, NJ, USA) with a 2.0-litre glass vessel containing 860 ml of medium stirred at *ca* 200 rpm, with a Watson Marlow 501U peristaltic pump as the outlet pump. The experiment with 50 g/l xylose feed was performed in a lab-scale vertical tubular bioreactor (working volume 610 ml) that was kept completely mixed as previously described (Dumsday et al., 1999; Martin et al., 2006).

All cultures were operated as chemostats at a dilution rate of 0.05/h–0.06/h with the temperature controlled at 30 °C and the pH at 6.0 by automatic addition of 3.0 M KOH. Sterilised antifoam solution (10 ml Dow Corning 1520 [Dow Corning, Midland, MI, USA] in 400 ml distilled water) was added manually as required. Constant volume was achieved by running the outlet pump faster than the feeding pump. Samples were removed by expelling liquid through the sampling line under nitrogen pressure. The bioreactor was inoculated with 10 ml of the inoculum culture and the fermentation was allowed to proceed as a batch for 8 h (at which time the population density was *ca* 2 g dry weight/l), after which the feed pump was switched on.

Ethanol in the fermentation broth was analysed by headspace gas chromatography as previously described (Hobley and Pamment, 1997).

2.5. Composition of RBS reagent solution

The rosanilin bisulphite (RBS) reagent was prepared by adding 0.2 ml rosanilin (consisting of 2.5 mg/ml basic fuchsin [C.I.42510, B.D.H., Germany] made up in 95% w/v ethanol) and 2.5 mg of sodium bisulphite (0.05 ml of a 50 mg/ml unsterilised solution) to 10 ml of sterilized modified Luria broth with no carbohydrate. The solution was stored in the dark at 4 °C. Although under these conditions it was found to be stable for up to 5 days (data not shown), it was prepared freshly before each use.

2.6. RBS assay procedure

Culture samples were removed from the bioreactor and the optical density of the suspension measured at 550 nm (OD_{550}). A known volume (e.g. 2 ml) of culture sample was then centrifuged for 5 min at 13,000 rpm (16,000*g*) in a biofuge pico microcentrifuge (Heraeus Instruments, Germany), washed in sterile Luria broth to remove residual acetaldehyde, recentrifuged, and the cells collected into 1 ml of sterilized Luria broth to make a final suspension with OD_{550} in the range 6.0–40.0. The sample (in a microtube) and 2 ml of RBS reagent in a glass cuvette were equilibrated separately in a water bath at 30 °C for 2 min, then mixed rapidly and the OD_{540} measured every 6–60 s (depending upon sample activity) against a distilled water blank using a Hitachi U-2000 spectrophotometer. Readings were continued for 2–20 min (depending upon the activity of the sample) and the net rate of acetaldehyde formation calculated as the maximum slope of the curve of OD_{540} versus time (see Section 3). The procedure was repeated for four different cell concentrations in the range OD_{550} 6.0–40.0 (each sample being concentrated to 1 ml as above) and the rate of change in OD_{540} plotted against cell concentration (in units of OD_{550}). From the slope of a straight line drawn through the above points, the activity of the sample in RBS assay units was expressed as $\Delta OD_{540}/(s.OD_{550})$.

3. Results

3.1. Development of the liquid phase RBS assay

The chemistry of the RBS assay is based on the aldehyde indicator plate assay devised by Conway et al. (1987) to identify high ethanol-producing colonies of a recombinant *E. coli* strain containing the *Z. mobilis pdc* and *adh* genes. The method employs Schiff's reagent, formed from pararosanilin and sodium bisulphite (Lillie, 1977). This produces an intense red colour in the presence of aldehydes and is a classic test for them (Vogel, 1989). In the method of Conway et al. (1987), chemically-mutated colonies of the strain were transferred to Luria agar plates containing ethanol and Schiff's reagent. Colonies which rapidly became red, due to microbial oxidation of ethanol to acetaldehyde, were assumed to be strong expressers of alcohol dehydrogenase B and thus likely also to be strong ethanol producers when a sugar rather than ethanol was used as the carbon source (i.e. the direction of the alcohol dehydrogenase reaction was reversed).

In the modification of the method in this work, microbial reduction of ethanol is carried out in liquid broth cultures and the rate of

red colour formation is quantified spectrophotometrically. Both ethanol and acetaldehyde can be assumed to diffuse freely and rapidly between the cells and the broth. This suggests that washed and concentrated whole cell samples, suspended in a suitable buffer with the colour reagent, should produce colour rapidly enough to provide a quantitative, indirect measure of ethanologenicity.

Reliable quantification of the rate of colour formation requires a suitable medium able to maintain cell viability while reacting only slowly if at all with components of the reaction mixture so that the coloured product remains stable. The stability of the coloured products was tested in both water and modified Luria broth lacking sugar by adding acetaldehyde (0.05 ml) to 3 ml of RBS reagent and monitoring the absorbance at 540 nm (the wavelength of maximum absorbance for the coloured product). In both water and modified Luria broth the colour developed almost instantaneously upon acetaldehyde addition (data not shown). The coloured product appeared completely stable in water and sufficiently stable in modified Luria broth. The final intensity was less in modified Luria broth, however this is not problematic as it is the relative rate of colour change, not the final magnitude that is important for the assay.

The assay was then tested for efficacy in the presence of *E. coli* KO11 cells, with acetaldehyde produced from the oxidation of ethanol by the cells' ADHII (data not shown). Using RBS reagent in distilled water alone, there was minimal development of red colour, even after more than 25 min of incubation. Upon subsequent addition of modified Luria broth (lacking sugar) there was an immediate, rapid development of the red colour and an increase in the OD_{540}. No colour development was observed in the absence of cells.

Based on the above, Luria broth was selected as the solution to be used in the further development of the assay. The reason for the beneficial effects of Luria broth on the development of the colour reaction could be due to the provision of non-carbon nutrients essential for metabolic functioning, but seems more likely to be a redox effect. In the absence of an exogenous source of carbon, it is unclear whether cellular metabolism of stored carbon would be sufficient to regenerate the NAD^+ used during ethanol oxidation by alcohol dehydrogenase. Luria broth contains tryptone and yeast extract which could be expected to contain compounds capable of re-oxidizing NADH under the test conditions.

3.2. Calculation of apparent ADH activity in RBS assay units

Recombinant *E. coli* KO11 contains the *adhB* gene from *Z. mobilis*, which encodes alcohol dehydrogenase II. ADHII catalyses both the production of ethanol from acetaldehyde and the reverse reaction. The latter will become the dominant one in sugar-free medium containing ethanol, especially if acetaldehyde is removed continuously via the Schiff's base formation reaction. The more strongly the strain expresses *adhB*, the faster the rate of acetaldehyde production and the faster the colour change. By monitoring spectrophotometrically the rate of colour formation, an empirical indication of the relative ADHII activity should be obtained.

For each determination of the RBS activity, four rate curves were obtained for four samples containing different volumes (and hence concentrations) of cell suspension, typically in the range 2–5 ml. The reaction rates $S_{1\text{-}4}$ were calculated as the maximum slopes of each curve of OD_{540} versus time (Fig. 1). During the period of maximum activity the OD_{540} versus time curves were normally linear over at least 60 s. The reaction rate S was calculated by linear regression from the linear central portion of the curve (the coefficient of correlation (r) was always better than 0.99).

The final estimate of the RBS activity was obtained as the slope of the straight line relating reaction rate (S_i, in OD_{540}/s) to sample volume (Fig. 2). For this purpose the sample volume (V_i) was converted to the equivalent cell concentration in OD_{550} and the final estimate of the RBS activity reported as RBS assay units (i.e. $\Delta OD_{540}/(s.OD_{550})$). While, for unknown reasons, this curve did not normally pass through zero, it was invariably linear as determined by linear regression analysis ($r > 0.99$) and thus provided a reliable empirical means of estimating the RBS activity.

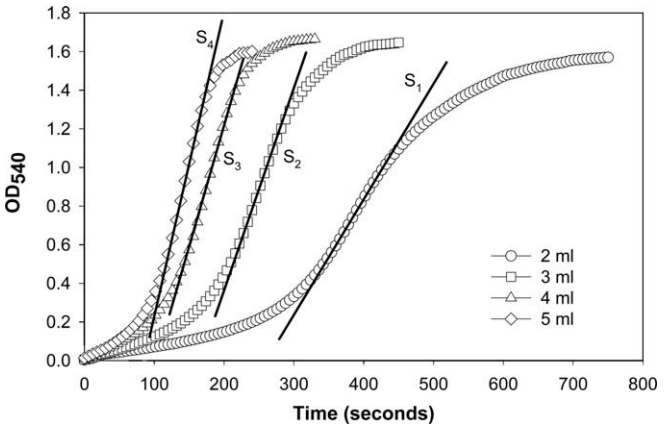

Fig. 1. Time-course of increase in OD_{540} during the RBS assay for a typical set of four curves from the RBS assay for culture samples of volumes 2, 3, 4 and 5 ml. The samples originate from a chemostat culture on 20 g/l xylose (shown by the black filled symbols in Fig. 4) and were removed at day 5, when the culture OD_{550} was 6.15. The activity of each sample in OD_{540}/s is given by the slope S_i. Each sample was concentrated to 1 ml by centrifugation prior to assay.

3.3. The RBS assay applied to continuous cultures of E. coli B ATCC 11303

Escherichia coli KO11 is derived from the *E. coli* B host strain ATCC 11303 (Ohta et al., 1991) which contains a native alcohol dehydrogenase that is expressed at very low levels compared to the transposed *Z. mobilis* gene (Ingram et al., 1987). In aldehyde indicator plate assays, colonies of ATCC 11303 formed some red colour, but much more slowly than KO11 colonies (data not shown). To determine to what extent acetaldehyde production from *adhE* would be detected by the new assay, and to provide a base-line for studies with *E. coli* KO11, the assay was tested in a

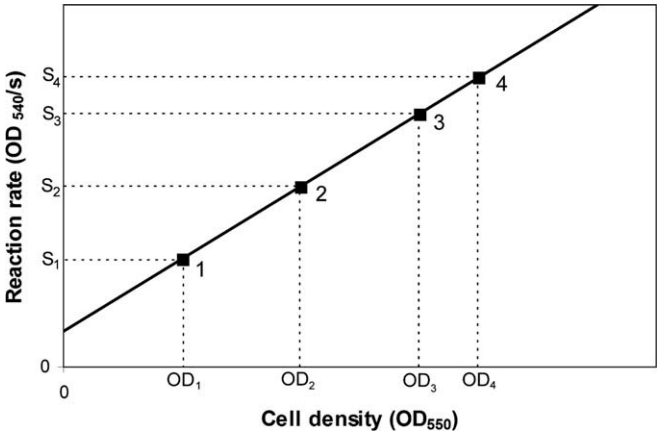

Fig. 2. Calculation of RBS activity. The RBS activity of the culture is determined as the slope of the straight line of reaction rate versus cell population density. For this purpose, the sample sizes, V_i, are re-expressed as cell population densities in units of OD_{550}.

continuous culture using ATCC 11303 with 2% xylose as substrate (Fig. 3).

The results show that strain ATCC 11303 has a measurable and approximately constant RBS activity of around 0.6–1.0 $\times 10^{-4}$ RBS assay units ($\Delta OD_{540}/(s.OD_{550})$). As discussed below, this is much lower than the RBS activities typically measured at the commencement of continuous fermentations by *E. coli* KO11 and should represent the base-line RBS activity for the strain when grown on xylose.

3.4. The RBS assay applied to continuous cultures of E. coli KO11

The RBS activities of recombinant *E. coli* KO11 during continuous fermentation were measured by the RBS assay in duplicate chemostat cultures with 2% glucose as the substrate (Fig. 3). In both experiments, the RBS activity was stable and constant at about 8.0 $\times 10^{-4}$ RBS assay units, consistent with the high and stable ethanol yield demonstrated previously for continuous culture of *E. coli* KO11 on glucose (Dumsday et al., 1997, 1999). Since the ethanol yields and productivities on glucose were the highest values obtained with strain KO11 in previous work (Dumsday et al., 1997, 1999), this suggests that an activity of *ca* 8.0 $\times 10^{-4}$ RBS assay units should be close to the maximum attainable with this strain.

The assay was then evaluated in two continuous culture experiments with 20 g/l xylose and a single experiment with 50 g/l xylose feed (Fig. 4). Previously, the KO11 strain was found to be phenotypically unstable when cultivated continuously on this sugar (Dumsday et al., 1999). In both experiments on 20 g/l xylose, the RBS activity dropped slightly from 9.4 $\times 10^{-4}$ RBS assay units at day 1 to *ca* 7.4 $\times 10^{-4}$ RBS assay units at day 5, then declined dramatically over about 3 days to *ca* 2.2 $\times 10^{-4}$ RBS assay units, finally stabilizing at 1.0–2.0 $\times 10^{-4}$ RBS assay units over the remainder of the experiments. For the experiment with 50 g/l xylose the RBS activity was initially lower than for the experiments on 20 g/l xylose but showed a similar decreasing trend. For all experiments, the time course of RBS activity closely mirrored the changes in ethanol yield, providing strong support for the efficacy of the assay as a measure of changes in ethanologenicity.

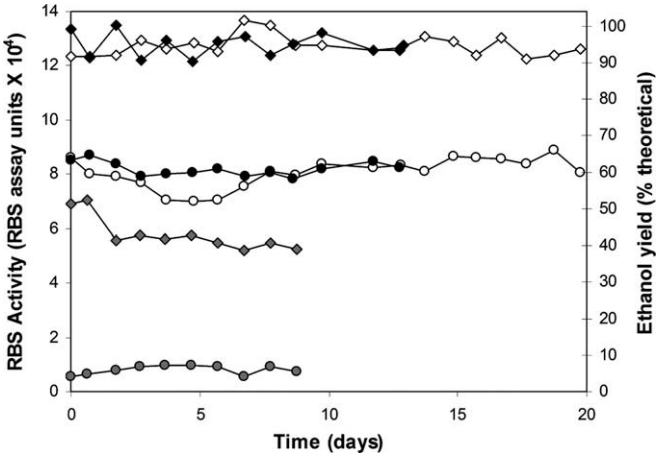

Fig. 3. RBS activity (●,○) and ethanol yield (♦,◇) of *E. coli* KO11 in duplicate chemostat cultures on modified Luria broth containing 20 g/l glucose. The RBS activity (●) and ethanol yield (♦) of the host strain ATCC 11303 grown on modified Luria broth with 20 g/l xylose is also shown. RBS activity at each time point was determined from four separate samples according to the procedure described above and is plotted as RBS assay units (i.e. $\Delta OD_{540}/(s.OD_{550})$).

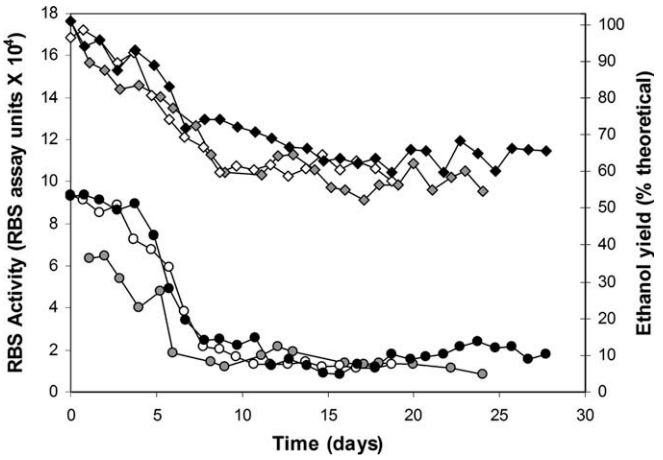

Fig. 4. RBS activity (●,○) and ethanol yield (♦,◇) of *E. coli* KO11 in duplicate chemostat cultures on modified Luria broth containing 20 g/l xylose and an additional experiment with 50 g/l xylose as feed (RBS activity ●; ethanol yield ♦).

4. Discussion

The above data show that the RBS assay is successful as an empirical indicator of changes in ethanologenicity during long-term continuous growth by *E. coli* KO11. The assay can typically be performed in less than 1 h, providing a much more rapid indication of any change in phenotype than can be obtained using conventional methods. Unlike conventional assays of alcohol dehydrogenase activity that measure the reduction of NAD^+ (e.g. Conway et al., 1987) the RBS method does not require cell lysis, making it more convenient to perform, while also avoiding the problem of NADH losses via NADH oxidase. Results from the assay mirror closely the trends in ethanol yield; including the stable yields when the organism is cultivated continuously on glucose (Fig. 3), and the contrasting fall in stability on xylose (Fig. 4). The assay also clearly reflects the much higher alcohol dehydrogenase activity of *E. coli* KO11 in comparison to its parent strain *E. coli* ATCC 11303 (Fig. 3). The overall correlation between ethanol yield and RBS activity can be demonstrated by assembling the total data points from all the experiments (Fig. 5).

The data show that hyperethanologenicity in *E. coli* KO11 (defined arbitrarily here as a yield greater than 80% of the theoretical maximum) is maintained over a wide range of RBS activities in the range 4.0–9.5 $\times 10^{-4}$ assay units. Below about 3.0 $\times 10^{-4}$ units the ethanol yield begins to drop substantially. During continuous culture experimentation, the observation of a falling RBS activity within this range may provide an early warning of the likely eventual loss of hyperethanologenicity of the culture, prior to its being detectable from the measured ethanol yield. Also, since the assay is based on ADH activity rather than direct measurement of ethanol yield, it should prove useful in diagnosing the cause of a low measured ethanol yield under particular experimental conditions i.e. whether a low yield is attributable to environmental factors such as inhibition or glucose repression rather than low ADH activity brought about by strain instability.

The occurrence of a measurable decline in RBS activity on xylose substrate, even in the first 5 days of the experiments, supports the conclusion reached in previous work (Dumsday et al., 1997, 1999) that the decline in hyperexpression of the *Z. mobilis* ethanologenic genes in *E. coli* KO11 starts from the very beginning of continuous anaerobic culture on xylose. This conclusion was previously drawn tentatively on the basis of the observed increase in acetic acid production at the beginning of the experiments (Dumsday et al., 1997, 1999), and is confirmed by the present evi-

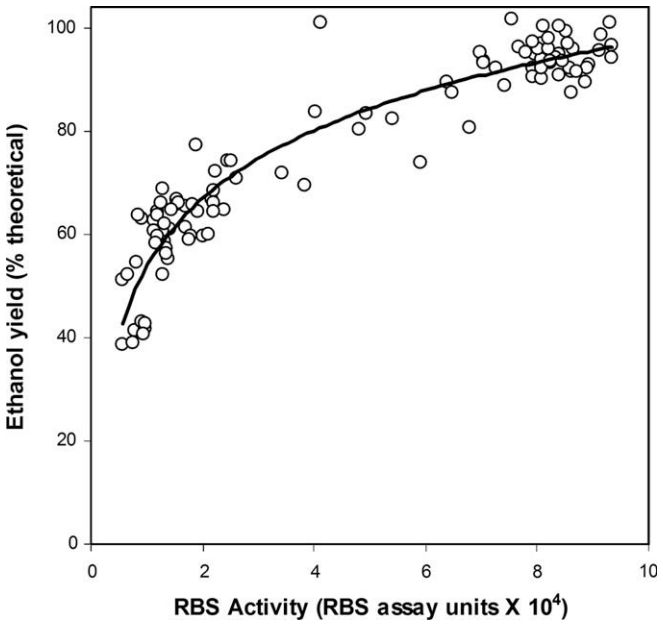

Fig. 5. Correlation between ethanol yield and RBS activity in *E. coli* KO11 and its parent strain during continuous culture experimentation with a logarithmic line-of-best-fit shown. Data are shown for all time points in all experiments in which the RBS activity was measured in Figs. 3 and 4.

dence. Compared to the value of under 1.0×10^{-4} RBS assay units obtained with the host strain (Fig. 3), the RBS activities at the end of the above two experiments still remained over 1.5×10^{-4} assay units, suggesting that the loss of expression of the transferred *adhB* genes was not quite complete. This accords with the ethanol yield that remains between 50% and 65% at the end of fermentation on xylose with *E. coli* KO11 but is only 45–50% for strain ATCC 11303.

5. Conclusions

The RBS assay appears to be an effective empirical method for rapidly ascertaining changes in ethanologenicity of *E. coli* KO11. The assay should also be applicable to other strains containing recombined *pdc* and *adh* genes for ethanol production.

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References

Beall, D.S., Ingram, L.O., 1993. Genetic engineering of soft-rot bacteria for ethanol production from lignocellulose. J. Ind. Microbiol. 11, 151–155.

Conway, T., Sewell, G.W., Osman, Y.A., Ingram, L.O., 1987. Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis*. J. Bacteriol. 169, 2591–2597.

Dien, B.S., Nichols, N.N., O'Bryan, P.J., Bothast, R.J., 2000. Development of new ethanologenic *Escherichia coli* strains for fermentation of lignocellulosic biomass. Appl. Biochem. Biotechnol., 181–196.

Dien, B.S., Cotta, M.A., Jeffries, T.W., 2003. Bacteria engineered for fuel ethanol production: current status. Appl. Microbiol. Biotechnol. 63, 258–266.

Doran Peterson, J., Ingram, L.O., 2008. Anaerobic respiration in engineered *Escherichia coli* with an internal electron acceptor to produce fuel ethanol. Ann. N.Y. Acad. Sci. 1125, 363–372.

Dumsday, G.J., Zhou, B., Buhmann, S., Stanley, G.A., Pamment, N.B., 1997. Continuous ethanol production by *Escherichia coli* KO11 in continuous stirred tank and fluidized bed fermenters. Australas. Biotechnol. 7, 300–303.

Dumsday, G.J., Zhou, B., Yaqin, W., Stanley, G.A., Pamment, N.B., 1999. Comparative stability of ethanol production by *Escherichia coli* KO11 in batch and chemostat culture. J. Ind. Microbiol. Biotechnol. 23, 701–708.

Galbe, M., Sassner, P., Wingren, A., Zacchi, G., 2007. Process engineering economics of bioethanol production. Adv. Biochem. Eng./Biotechnol. 108, 303–327.

Gomez, L.D., Steele-King, C.G., McQueen-Mason, S.J., 2008. Sustainable liquid biofuels from biomass: the writing's on the walls. New Phytol. 178, 473–485.

Hahn-Hägerdal, B., Jeppsson, H., Olsson, L., Mohagheghi, A., 1994. An interlaboratory comparison of the performance of ethanol-producing microorganisms in a xylose-rich acid hydrolyzate. Appl. Microbiol. Biotechnol. 41, 62–72.

Hobley, T.J., Pamment, N.B., 1997. Liquid injection gas chromatography and the ALDH assay overestimate free acetaldehyde in complex fermentation media. Biotechnol. Tech. 11, 39–42.

Ingram, L.O., Conway, T., 1992. Cloning and sequencing of the alcohol dehydrogenase II from *Zymomonas mobilis*. U.S. Patent 5,162,516.

Ingram, L.O., Conway, T., Clark, D.P., Sewell, G.W., Preston, J.F., 1987. Genetic engineering of ethanol production is *Escherichia coli*. Appl. Environ. Microbiol. 53, 2420–2425.

Ingram, L.O., Alterthum, F., Ohta, K., Beall, D.S., 1990. Genetic engineering of *Escherichia coli* and other enterobacteria for ethanol production. In: Pierce, G.E. (Ed.), Developments in industrial microbiology. Elsevier Science Publishers, New York, pp. 21–30.

Jarboe, L.R., Grabar, T.B., Yomano, L.P., Shanmugam, K.T., Ingram, L.O., 2007. Development of ethanologenic bacteria. Adv. Biochem. Eng./Biotechnol. 108, 237–261.

Jeffries, T.W., 2006. Engineering yeasts for xylose metabolism. Curr. Opin. Biotechnol. 17, 320–326.

Keshav, K.F., Yomano, L.P., An, H., Ingram, L.O., 1990. Cloning of the *Zymomonas mobilis* structural gene encoding alcohol dehydrogenase I (*adhA*): sequence comparison and expression in *Escherichia coli*. J. Bacteriol. 172, 2491–2497.

Lillie, R.D., 1977. H.J. Conn's biological stains, ninth ed. The Williams and Wilkins Co., Baltimore.

Lima, K.G., Takahashi, C.M., Alterthum, F., 2002. Ethanol production from corn cob hydrolysates by *Escherichia coli* KO11. J. Ind. Microbiol. Biotechnol. 29, 124–128.

Martin, G.J.O., Knepper, A., Zhou, B., Pamment, N.B., 2006. Performance and stability of ethanologenic *Escherichia coli* strain FBR5 during continuous culture on xylose and glucose. J. Ind. Microbiol. Biotechnol. 33, 834–844.

Ohta, K., Beall, D.S., Mejia, J.P., Shanmugam, K.T., Ingram, L.O., 1991a. Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl. Environ. Microbiol. 57, 893–900.

Ohta, K., Beall, D.S., Mejia, J.P., Shanmugam, K.T., Ingram, L.O., 1991b. Metabolic engineering of *Klebsiella oxytoca* strain M5A1 for ethanol production from xylose and glucose. Appl. Environ. Microbiol. 57, 2810–2815.

Rao, K., Chaudhari, V., Varanasi, S., Kim, D.-S., 2007. Enhanced ethanol fermentation of brewery wastewater using the genetically modified strain *E. coli* KO11. Appl. Microbiol. Biotechnol. 74, 50–60.

Vogel, A.I., 1989. Vogel's Textbook of Practical Organic Chemistry, fifth ed. Wiley, New York.

Yomano, L.P., York, S.W., Ingram, L.O., 1998. Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J. Ind. Microbiol. Biotechnol. 20, 132–138.

Zhou, B., Martin, G.J.O., Pamment, N.B., 2008. Increased phenotypic stability and ethanol tolerance of recombinant *Escherichia coli* KO11 when immobilized in continuous fluidized bed culture. Biotechnol. Bioeng. 100, 627–633.