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Development of tolerance to aldehyde-based inhibitors of pretreated lignocellulosic biomass sugars in *E. coli* MG1655 by sequential batch adaptive evolution

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Abstract

Aim: The current study involved carrying out adaptive evolution to inculcate tolerance to hydrolysate-derived aldehyde-based inhibitors, furfural, vanillin, syringaldehyde and 4-hydroxybenzaldehyde (4-HB) for the valorization of pretreated lignocellulosic biomass.

Methodology: The growth-inhibitory effects of the aforementioned inhibitors on *E. coli* MG1655 were investigated. The percentage of inhibition was calculated from the initial growth, followed by extrapolating the IC_{50} values for each inhibitor. Based on these findings, adaptation experiments were conducted for individual inhibitors at a concentration lesser than or closer to IC_{50} .

Results: The specific growth rate of cells was lowered by 2.2-, 3-, 1.3- and 5- fold when grown in the presence of furfural, vanillin, syringaldehyde and 4-hydroxybenzaldehyde (4-HB), respectively. The adapted strains which were grown in the presence of furfural (9mM), vanillin (9mM), syringaldehyde (8mM) and 4- HB (6mM) individually showed around 1.5 -2.5- fold increase in the specific growth rate as compared to the wild-type with decreased lag phases and increased final cell densities.

Interpretation: The strains, subjected to adaptive evolution, resulted in increased tolerance to single inhibitors and these will further be sequentially adapted to other three inhibitors for their utilization in the valorization of pretreated lignocellulosic biomass.

Key words: Adaptive evolution, Aldehyde- based inhibitors, *E. coli*, Inhibitor tolerance, Specific growth rate

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Introduction

The growing concerns of environmental pollution and the potential depletion of non-renewable sources have made scientists to discover sustainable microbial processes for the production of chemicals and fuels utilizing inexpensive and abundant feedstock (Namita *et al.*, 2015). One such feedstock that fits the criteria is lignocellulosic biomass which is made up of tightly interlinked and intra-linked units of cellulose, hemicellulose and lignin as its major components. Several non-consumable materials such as bagasse, corn stover, wheat straw and rice husk, which are abundant, have also been used as a feedstock for microbial fermentation (Sánchez and Cardona, 2008; Balakrishnaraja *et al.*, 2017). A significant drawback of using these resources as a microbial feedstock is mandatory requirement of pretreatment to overcome the inherent hydrolytic stability conferred by the recalcitrant materials, resulting in inevitable formation of degradation products such as furan aldehydes (furfural) and phenolic aldehydes (vanillin, 4-hydroxybenzaldehyde and syringaldehyde) that are detrimental to microbial growth and productivity (Jönsson and Martín, 2016).

Though several detoxification methods have been reported, generation of toxic byproducts in case of harsh chemical detoxification and long incubation times, loss of sugars and comparatively higher costs of biological detoxification methods impede the practical usability of lignocellulosic biomass. Hence, there arose a need to explore targeted genome engineering and evolutionary adaptation strategies to develop a strain tolerant to inhibitors in the hydrolysate. Reports on genome engineering of microbes to impart inhibitor tolerance by over-expression of enzymes are sparse because of the difficulty in understanding the mechanism of stress tolerance in microbes (Liu *et al.*, 2004). Random mutagenesis and genome shuffling are few methods which impart inhibitor resistance without the need to know the set of genes to be modified to effect beneficial mutations (Chandel *et al.*, 2013; Bajwa *et al.*, 2009). Some researchers suggest that the generation of inhibitor-tolerant strains using adaptive evolution has proved to be more successful than the above-mentioned genetic engineering strategies (Kurosawa *et al.*, 2015).

Adaptive evolution of a strain imparts an improved inhibitor tolerance by iterative genetic diversifications and a process-relevant selection procedure in the presence of an inhibitor as selection pressure. Many process strategies for adaptation of cells in the presence of inhibitors have been reported, which include batch cultivations to achieve high cell densities, cell-retention cultivation using immobilized cells or cell-recirculation (Almeida *et al.*, 2007). Long term adaptation experiments have been conducted either as a continuous chemostat system by maintaining very low dilution rates or in a sequential batch culture where cells would grow at μ_{max} (Smith *et al.*, 2014). *Escherichia coli* is one of the most widely used host strains in applied biotechnology for producing variety of microbial metabolites, including recombinant proteins (Waegeman and De,

2012), biofuels (Trinh and Srienc, 2009), and bio-products (Centeno *et al.*, 2014). From industrial perspective, it offers several advantages over other commonly used organisms for bioprocesses, which include a very low doubling time of approximately one hour in defined media (Zaldivar *et al.*, 1999), ability to ferment both hexose and pentose sugars, availability of extensive knowledge about the *E. coli* genome and regulation and extensive molecular tools (Idalia and Bernardo, 2017). Several protocols have already been established and optimized for different bio products with high turnover (Koppolu and Vasigala, 2016). These qualities make *E. coli* a lucrative platform for producing a versatile array of bio products from lignocellulosic biomass sugars cost-effectively.

In this study, *E. coli* MG1655 strains were developed individually tolerant to four most commonly found aldehyde-based inhibitors in the hydrolysate: furfural, vanillin, syringaldehyde and 4-hydroxybenzaldehyde (4-HB). The concentration-dependent effect of these inhibitors on *E. coli* growth was assessed. Based on the inhibitory performance, strains with improved tolerance against the inhibitors were generated by the adaptive evolution approach. The aim of adaptive evolution is to improve the microbial growth rate, cell densities and shorten the lag phase in the presence of inhibitors for its use in pretreated biomass hydrolysates. Further, tolerance to other inhibitors may be made inherent by protoplast fusion or genome shuffling of strains tolerant to individual inhibitors or by subsequent adaptive evolution of these strains to other inhibitors. The objective of this study is to develop *E. coli* that is tolerant to the inhibitors in biomass hydrolysate, eventually resulting in efficient sugar utilization.

Materials and Methods

Microbial strain and culture media: The parent strain, *E. coli* MG1655 was maintained as glycerol stock in culture medium containing 25% glycerol at -80°C. The tolerance studies and evolution experiments were carried out at 37°C using 50 ml of chemically defined minimal salts (M9) medium (Pardee *et al.*, 1959), with 4g l⁻¹ of glucose as carbon source, supplemented with inhibitors at various concentration, in 250 ml flask. The salts were autoclaved separately while trace elements and inhibitors were filter sterilized using 0.2µm nylon filters. These sterile media components were mixed prior to inoculation. M9 medium without any inhibitors was used as a control for the study.

Tolerance studies in shake flask: To study the inhibitory effects of various inhibitors, 49.5 ml of fresh M9 medium was aseptically added in a 250 ml conical flask, followed by the addition of inhibitor at defined range of 2-10 mM for furfural, 2.5-12.5 mM for vanillin, 2-10 mM for syringaldehyde and 2-7 mM for 4-hydroxybenzaldehyde. Each flask was inoculated with 1% aliquots of overnight-grown culture. The flasks were incubated in an orbital shaker at 180 rpm and 37°C. The growth curves were determined by measuring the OD_{600nm} of samples from each flask every hour. One unit of OD_{600nm} corresponds to 0.3 g l⁻¹ of dry cell

weight of *E. coli*. The IC_{50} values for the inhibitors were calculated by plotting a graph between the percentages of inhibition, which was calculated by taking the OD_{600nm} values at the exponential phase of cell growth, versus the concentration of each inhibitor. A trend line was inserted to the graphs and the IC_{50} values of each inhibitor were taken as the concentration at which the percent inhibition was 50.

Adaptive evolution of the wild type *E. coli* MG1655: A long-term evolutionary laboratory adaptation protocol for *E. coli* was developed using the IC_{50} values calculated from the previous observations to establish tolerance to inhibitors. To 50 ml of M9 medium in a 250 ml conical flask, 0.1% of overnight culture was aseptically inoculated. The initial sub-lethal concentration of each inhibitor was used and the flasks were kept in a shaker incubator at 37°C, 180 rpm. Passages were made periodically at every 24-hr time interval by monitoring the cell growth. When the OD_{600nm} of the sample was same or lesser than the previous passage, the cells were transferred iteratively to a medium containing same concentration of inhibitor. Once the strain had adapted to this concentration, it was then transferred to the medium containing a higher concentration of inhibitor and the same process was repeated till the strain got adapted to the desired concentration of inhibitor. After the adaptive evolution, the culture was streaked on M9 agar plates with inhibitors for isolated colonies. Glycerol stock cultures of adapted strains

were prepared in culture medium with 25% glycerol and the adapted concentration of inhibitor and maintained at -80°C.

Evaluation of microbial adaptation in inhibitory media: To verify the effect of adaptation, comparative experiments were carried out on three independent populations. The adapted strain and wild-type strain were grown in 1% inoculum of M9 medium taken in 250 ml conical flasks containing concentration of inhibitors that should be detrimental for the growth of wild-type strain. The native strain was inoculated without inhibitors and was used as control. The flasks were kept in a shaker incubator at 37°C, 180rpm. Absorbance was read periodically every hour for 8 hrs. Time versus OD_{600nm} was plotted to determine the effect of each inhibitor on the *E. coli* MG1655 strain.

Statistical analysis: The experiments were performed in duplicates and the results were expressed as mean. Standard deviations in these experiments varied between 0.001 and 0.042.

Results and Discussion

To understand the effect of aldehyde-based inhibitors on various growth parameters, tolerance studies of *E. coli* MG1655 in the presence of vanillin, 4-HB, furfural and syringaldehyde individually were performed (Fig. 1). At first, the cells were grown in the presence of varying concentrations of vanillin, 4- HB,

Fig. 1: Relationship between the growth of *E. coli* MG1655 and the concentration of (A) Vanillin; (B) 4-HB; (C) Furfural and (D) Syringaldehyde in the medium. (Standard deviation among the duplicates were below 0.008).

Fig. 2: Inhibitory effects of Vanillin, 4- HB, Furfural and Syringaldehyde on the growth of *E. coli* MG1655 (Standard deviation among the duplicates were below 0.005).

furfural and syringaldehyde in the defined medium as stated in the materials and methods which was chosen based on the amount of inhibitors found in the biomass hydrolysates (Rumbold *et al.*, 2009; Sannigrahi *et al.*, 2010; Huang *et al.*, 2011; Wilkinson *et al.*, 2016; Du *et al.*, 2010) along with control experiment without inhibitors. The amount of inoculum used in all these cultures was 0.1% and therefore no lag phase was found to exist in the control experiment and also in some of the cultures with inhibitors as well. The specific growth rates of all the cultures were monitored. In the control experiments, during early logarithmic phase, the cells grew exponentially and later started to decrease marginally either due to substrate depletion or product inhibition. In the case of vanillin and 4-HB, no lag phase was seen and the specific growth rate decreased with increasing concentration of inhibitors during the log phase of the culture. These results depict the influence of inhibitor concentration on the growth rate with almost no growth leading to complete inhibition for vanillin and 4-HB at 12.5 and 10mM, respectively. Vanillin resulted in around 2.1- and 3- fold reduction in specific growth rates at 7.5 and 10 mM concentrations, respectively, while, 4-HB resulted in 2.8- and 5- fold decline in specific growth rates at 6 and 8 mM concentrations, respectively, as compared to the control experiment.

As far as furfural was concerned, in the lag phase, cell lysis was found to occur with decreasing cell densities and later the cells got adapted. Reports hypothesize that the adaptation process could take place during the extended lag phase before the recovery of active cell growth and metabolism (Liu *et al.*, 2004). The specific growth rate decreased with increasing inhibitor concentration having 1.8- and 2.2- fold lower specific

growth rates, at a furfural concentration of 6 and 8mM, than that of the control experiment. In the case of syringaldehyde, no lag phase was seen at lower concentrations of inhibitors. However, with increasing concentration of inhibitor, the lag phase was observed for 2 hrs indicating that cells took more time to enter the log phase. Further, during the early log phase, the specific growth rate was decreased with increasing concentration of inhibitor. During the mid-log phase, the specific growth of the cultures improved indicating that the adaptation took place and almost matched with that of the control experiment. Therefore, syringaldehyde resulted in only 1.3- fold reduction in the specific growth rate of cells at a higher concentration of 10mM when compared to that of control. Further, in the later phase, a decrease in growth due to the culture conditions as seen in the control experiment was observed. The reduction of both the growth rates and cell densities suggests that both the stoichiometry and the rate of conversion of substrates to cellular material were affected in the presence of inhibitors (Franden *et al.*, 2013).

This decreased growth capacities of microbial cells, at higher inhibitor concentrations, could be connected to the expenditure of an increased fraction of total energy on cell maintenance in the presence of inhibitors (Zha *et al.*, 2012). A similar observation was found in yeasts, where the addition of furfural resulted in altered intracellular NAD(P)H and ATP levels contributing to negative effects on energy generating glycolytic and TCA fluxes along with decreased activity/ inhibition of glycolytic enzymes and other dehydrogenases (Almeida *et al.*, 2007, Sjulander and Kikas, 2020). Furan aldehydes (furfural) and phenolic aldehydes (vanillin, 4-HB and syringaldehyde) have

Fig. 3: Adaptive evolution of *E. coli* MG1655 in a defined medium containing a particular concentration of (A) Vanillin; (B) 4-HB; (C) Furfural and (D) Syringaldehyde. The number above the bar/ column indicates the number of passages or days of transfer and the arrow mark indicates transfer to a higher concentration of inhibitor (Standard deviation among the duplicates were between 0.003 and 0.042).

been reported to hamper necessary metabolic processes by competing with the enzyme active site or depleting the nutrients or cofactors necessary for enzymatic activity (Mills *et al.*, 2009). Based on the results obtained from the inhibitor studies, the percentage of inhibition exerted by each inhibitor at various concentrations was calculated (Fig. 2). This was further used to calculate the IC_{50} value of each inhibitor by graphical method. From the plot obtained, it was observed that growth kinetics does not linearly correlate with the inhibitor concentration, but rather becomes static at higher concentration. However, the points were fitted into a linear equation for two reasons: the extrapolated values were approximately equal to the actual values of growth ($R^2 > 0.95$) and since the extrapolated values were slightly higher than the actual values, it would be more useful to adopt them for the adaptive evolution experiment. Therefore, with this slight approximation, the IC_{50} values of vanillin, 4-HB, furfural and syringaldehyde were calculated to be 5.3mM, 3.4mM, 4.3mM and 5.7mM, respectively. From the IC_{50} values, the growth inhibition of aldehydes on *E. coli* cells, listed in decreasing order of magnitude, was 4-HB > furfural > vanillin > syringaldehyde.

The mechanism of inhibition of phenolic aldehydes has been reported to be the loss of membrane integrity by altering its protein to lipid ratio (Jönsson *et al.*, 2013) and also affecting the other hydrophobic sites within the cell as well (Zaldivar *et al.*, 1999). The toxicity of phenolic aldehydes in our study was also found to directly correlate to low molecular weight, ortho-substituent position and high hydrophobicity with the order of inhibition exerted by these phenolic aldehydes on *E. coli* MG1655 similar to that reported by Zaldivar *et al.* (1999). Based on the calculated IC_{50} value, the initial sub-lethal concentration of inhibitor for the adaptation process was found to be lower than or close to the IC_{50} for the respective inhibitor. The requirement at the end of the adaptation process was a strain with superior growth capacities having improved growth rates, higher final cell densities and lack of lag phase than the wild-type when grown in the presence of inhibitors. To conduct an efficient adaptation and selection regime for improved fitness of *E. coli* towards aldehyde-based inhibitors, two key methods were adopted. Firstly, step-wise adaptation for a single inhibitor at a time was done since the adaptation of cells to a combination of inhibitors was reported to impose significant growth suppression (Kurosawa *et al.*, 2015). Secondly, late log phase cells were exposed to a relatively higher concentration of inhibitors along with low inoculum size to ensure substantial selection pressure.

Additionally, the cells exposed to a prolonged stationary phase before serial transfer were reported to show rapid evolutionary adaptation. These factors were incorporated in the selection regime for establishing a dynamic environment for the microbial population to acquire selective advantage for survival (Jönsson *et al.*, 2013; Dragosits and Mattanovich, 2013) thereby ensuring the dominance of cells which showed improved tolerance to inhibitor. Thus, the adaptation experiment was initiated by inoculating 0.1% of *E. coli* MG1655 individually, in the defined medium containing 4mM, 4mM, 3mM and 5mM of

vanillin, 4-hydroxybenzaldehyde, furfural and syringaldehyde respectively, for adaptation (Fig. 3). The cells were sequentially adapted to a vanillin concentration of 9mM in 8 phases. In the initial phase, the cells took around 6 passages to adapt to the concentration of 4.5mM. However, at higher concentrations, the cells gained improved fitness in around 5-6 days. The final cell density on day 48 was 1.9-fold higher than that of day 1, and hence the adaptation was stopped at this concentration.

In the case of 4-HB and syringaldehyde, the cells were sequentially adapted to a final concentration of 6 and 8mM in 3 and 4 phases, respectively. In both cases, the tolerance was achieved in just 4-6 passages in each concentration. The adaptation process was terminated for the inhibitors, 4-HB and syringaldehyde, at day 12 and day 19 when 2.8- and 2.3- fold higher final cell densities than that on day 1 were obtained respectively. In the case of furfural, the cells were adapted to a final concentration of 10 mM in 8 phases. In the initial phase, the cells struggled for 8 and 16 passages to get acquainted with the initial concentration of 3 and 4mM, respectively. Further adaptation caused rapid shortening of lag phase, with 3-5 passages to get accustomed to a particular concentration of furfural and a significant increase in the cell densities after 40 passages. The final cell density at day 48 was 4-fold higher than that of day 1, and hence the adaptation experiment was terminated. In all the cases, the cells struggled for initial passages and later gained fitness to survive in the presence of inhibitor. Particularly, in the presence of furfural, growth rate started to improve only after 40 passages as opposed to syringaldehyde which required only limited passages to achieve a positive growth rate.

In addition to this, an increased lag phase with a decreased final cell-density was observed when the cells were grown in the presence of inhibitors, as compared to the control strain. The prolonged lag phase during the initial adaptation experiment might be attributed to the dominance of non-beneficial mutations for survival where maximum cell adaptation process could occur (Smith *et al.*, 2014). This is followed by increased beneficial mutations contributing to the synthesis of new enzymes and cofactors, and in doing so recovering its active cell metabolism (Huang *et al.*, 2009) and growth by conversion of aldehydes to less toxic corresponding alcohols or acids (Boguta *et al.*, 2014; Miller *et al.*, 2009). Particularly, in the case of furfural, the increased flux through pentose phosphate pathway has been implied to be the most probable reason for the tolerance of cells to this inhibitor (Gorsich *et al.*, 2006).

This observation also coincides with the reports which suggest that over-expression of enzymes like alcohol dehydrogenase, pentose phosphate pathway enzymes, phenyl acrylate decarboxylase and laccase have been reported to impart resistance to inhibitors in hydrolysate (Heer and Sauer, 2008; Kim and Hahn, 2013; Parawira and Tekere, 2011; Larsson *et al.*, 2001). Over-expression of genes encoding reductase enzymes has also been reported to improve tolerance to 4-hydroxybenzaldehyde and vanillin (Yang *et al.*, 2018). It has been

Fig. 4: Comparison of the growth of wild-type and adapted *E. coli* MG1655 in the presence of (A) 9 mM Vanillin; (B) 6 mM 4-Hydroxybenzaldehyde; (C) 9 mM Furfural and (D) 8 mM syringaldehyde. *E. coli* MG1655 grown in media without any inhibitor was used as control (Standard deviations among the duplicates were between 0.001 and 0.007).

Fig. 5: Level of inhibition of Vanillin, 4- HB, Furfural and Syringaldehyde on the growth of adapted and wild-type strain. *E. coli* MG1655 grown in absence of inhibitors was used as control (Standard deviation among the duplicates were below 0.005). The value on top of each bar indicates the specific growth rate of that species in h^{-1} .

reported that adaptation using repeated batch process with replacement of fresh medium containing new load of inhibitors forced the cells to become tolerant owing to constant exposure of these cells to non-reduced form of inhibitors (Wallace and Gorwa, 2013). Further to evaluate the tolerance of developed strains, the adapted and wild-type *E. coli* were grown in the presence of final adapted concentration of vanillin, 4- HB, furfural and syringaldehyde, in a defined medium as stated in materials and methods along with the control, *E. coli* MG1655, grown in absence of an inhibitor. The amount of inoculum used in all these cultures was 1% and, in each case, the specific growth rates, length of lag phase and final cell densities achieved were monitored. Each adapted population displayed an increase in growth rate over the wild-type strain in the presence of an inhibitor (Fig. 4).

In the case of vanillin, 4- HB, furfural and syringaldehyde individually, no lag phase was observed with the adapted strains and they exhibited a 1.7-, 2.5-, 2.2- and 1.5-fold increase in the specific growth rates with a corresponding 3.3-, 5.4-, 3.1- and 1.3-fold higher cell densities than the wild- type grown in the respective inhibitors. However, the final cell densities of adapted strain were lower than the control strain which could be considered as a trade-off event, for the utilization of low-cost pretreated lignocellulosic biomass (Wallace and Gorwa, 2013). Interestingly, specific growth rates of adapted strains in the presence of inhibitors were nearly close to that of the control experiment which shows the clear advantage of evolutionary adaptation resulting in a sugar utilization rate almost close to that of the cells grown using non- inhibitory carbon sources. In a similar study, fermentation of both glucose and xylose in the

hydrolysate was faster for the adapted *Saccharomyces cerevisiae* when compared to non-adapted one with higher ethanol specific and volumetric productivities (Martin et al., 2007). In contrast to traditional and molecular biological approaches, adaptive evolution improves inhibitor tolerance even in the absence of knowledge on the complex interaction of inhibitors with the biochemical network (Bajwa et al., 2010). The potential of adaptive evolution proves to be vital in improving inhibitor tolerance by allowing cell variants with selective advantage to exponentially take over the initial inhibitor- intolerant cell population. The estimation of percentage of inhibition exerted by the adapted concentration of inhibitors on the growth of adapted and wild- type *E. coli* was performed. The performance of adapted strains was exceptional with 1.7-, 2.2-, 8.7- and 2.4- fold decline in the percentage of inhibition exerted by vanillin (9mM), 4-HB (6mM), furfural (9mM) and syringaldehyde (8mM), respectively, when compared to wild- type *E. coli* (Fig. 5) proving further that the cells have got completely accustomed to the inhibitory environment (Kurosawa et al., 2015) and have gained fitness to be subjected for further adaptation to a combination of inhibitors thereby developing a tolerant *E. coli* MG1655 which could be efficiently utilized for the valorization of lignocellulosic biomass sugars. Studies suggest that one of the mechanisms which incorporate tolerance to inhibitors like furfural could be modulation of redox machinery (Jilani et al., 2020) or improved ability of MDR efflux pumps, porins, and other selected transporters in *Escherichia coli* (Kurgan et al., 2019).

Also, such single inhibitor tolerant strains have been reported to be necessary for the elucidation of mechanisms of

stress tolerance (Liu *et al.*, 2005). The development of inhibitor tolerant *E. coli* will have immense scope in industrial production of therapeutic proteins as well as value added chemicals by utilizing sugars in the lignocellulosic biomass. In this study, *E. coli* MG1655 individually tolerant to aldehyde-based inhibitors, furfural, vanillin, syringaldehyde and 4-HB have been developed which had improved specific growth rates and higher final cell densities as compared to the wild-type strain when grown in the presence of inhibitors. These strains, which were adapted to a single inhibitor, will further be subjected to adaptation to other three inhibitors for improved production of bio products by utilizing sugars from pretreated lignocellulosic biomass.

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Add-on Information

Authors' contribution: G. Padmapriya: Study conception, experimental design, conducted experiment, data collection, analysis and manuscript drafting; V. Dhivya: Conducted experiment, data collection and analysis; M. Vishal: Manuscript drafting; Y.A.J. Roshni: Conducted experiment and manuscript review; T. Akila: Manuscript review; S. Ramalingam: Study conception, experimental design and manuscript review.

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