Enhancement of Xylose Utilization in Various *Escherichia coli* Strains Through Adaptive Laboratory Evolution (ALE) Experiments

Yousef Vatanparast[1], Gholamhossein Ebrahimipour[1], Mohammad Yaghoubi-Avini[1*]

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Abstract

In today's industrial landscape, utilizing renewable energy sources, such as biomass, rather than non-renewable resources like fossil fuels poses a significant challenge for various chemical production processes. lignocellulose biomass is one of the abundant biomass sources, and xylose, the second most common sugar in nature, accounts for an average of 24% of the sugars in hydrolyzed lignocellulose. Xylose emerges as a promising renewable source for biofuels and chemical production. Recent studies have highlighted the significant potential of *Escherichia coli* for biofuels and valuable chemical production through the metabolism of D-xylose. The most extensively researched methods for enhancing xylose uptake and metabolism in *E. coli*. This study evaluated four *E. coli*, K12, DH5α, BL21, and BW25113 strains under identical conditions of adapting cells to growth on the AM1 medium with glucose and subsequently three aerobic subcultures in an AM1 medium containing 2 g/L xylose for adaptive laboratory evolution experiments. To accurately compare the adaptation of each strain, the growth curve was plotted using a wavelength of 600 nm, and logarithmic phase-specific growth rate (μ) was calculated. The findings showed that the *E. coli* DH5α strain had the highest adaptation to aerobic conditions with low xylose concentrations compared to other strains. In contrast, the *E. coli* BL21 demonstrated minimal adaptation to xylose consumption under the defined conditions. Thus, strains undergo different evolutionary paths under identical conditions, and some adapt better. These findings can contribute to improving the production of biofuels and chemicals from xylose.

Keywords: Adaptive Laboratory Evolution (ALE), Xylose, *E. coli*, Specific growth rate, Growth curve

Introduction

Increasing global concerns over the energy crisis and climate change have made the development of clean and sustainable resources for fuel and chemical production crucial (Chukwuma et al., 2021). Lignocelluloses' biomass, which is abundant and non-food based, is an environmentally friendly alternative to fossil fuels. It consists mainly of cellulose (30-50%), hemicellulose

1-Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran
*Corresponding author email: m_yaghoubi@sbu.ac.ir
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(25-30%), and lignin (15-20%), with cellulose being a homopolymer of glucose and hemicellulose primarily composed of xylose, arabinose, and galactose (Antolini 2021; Kim 2018). A significant challenge is that most fermentative microorganisms cannot effectively metabolize lignocellulose-derived products other than glucose (Wang et al., 2022). Xylose, the second most abundant sugar in nature, represents 18 to 30% of sugars from lignocellulose and is a promising renewable resource for biofuels and chemicals (Rao et al., 2023; Narisetty et al., 2022). However, improving xylose uptake efficiency and establishing effective metabolic pathways are essential for enhancing fuel and chemical production from xylose (Zhao et al., 2020; Sun and Jin 2021).

Escherichia coli is a promising host for converting D-xylose into valuable compounds, primarily absorbing it through a high-affinity transporter (XylFGH) encoded by the *xylFGH* gene (Bañares et al., 2021). Additionally, a low-affinity proton symporter (*xylE*) can partially uptake D-xylose, and arabinose transporters (AraFGH and AraE) can import D-xylose with lower efficiency (Onyeabor et al., 2020; Wang 2020). Once inside the cell, D-xylose is isomerized to D-xylulose by xylose isomerase (XI or XylA) and then phosphorylated to D-xylulose-5-phosphate by xylose kinase (XK or XylB), entering the pentose phosphate pathway (Jacob et al., 2023; Saxena et al., 2023). The transcription of the *xylFGH* and *xylAB* genes in *E. coli* is regulated by the cyclic AMP receptor protein (CRP) and the XylR regulator (Zhu et al., 2022; Schubert and Unden 2021). The expression of the *xylFGH*, *xylE*, and *xylAB* genes is positively regulated by XylR in response to xylose through direct binding to their promoters (Barthe et al., 2020). Overall, these regulatory mechanisms are crucial for optimizing D-xylose metabolism in *E. coli*, facilitating its use as a renewable resource for bioconversion processes (Yin et al., 2021).

Adaptive laboratory evolution (ALE), directed evolution, and rational design are powerful tools for improving various processes in microorganisms (Wu et al., 2022; Qu et al., 2020). It includes growth rate optimization, tolerance increase, substrate utilization, and product formation (Reetz 2022; Wu et al., 2022). ALE technique mimics natural selection in laboratory cultures by applying selective pressure to select phenotypically improved strains through adaptive mutation accumulation (Sandberg et al., 2019). Studies indicate that by modifying growth conditions and selective pressures, various genes involved in xylose uptake and metabolism can be influenced and altered (Domingues et al., 2021). Therefore, using adaptive laboratory evolution (ALE) and directed evolution to improve the utilization of xylose substrates with or without glucose has yielded significant results (Wang et al., 2023; Wang et al., 2021).

In this study, the growth rate of four commonly used *E. coli* strains was investigated aerobically by repeated culture technique under a low xylose concentration as the sole carbon source. The results demonstrated that different strains of the

same species follow distinct evolutionary trajectories under identical conditions, and some strains exhibit superior adaptation. This understanding is crucial for optimizing microbial growth in biotechnological applications.

Material and methods

Culture media

LB (Luria Broth) medium containing 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl was used for strains preculturing (MacWilliams and Liao, 2006). AM1 medium containing 2.63 g/l $(NH_4)_2HPO_4$, 0.87 g/l $NH_4H_2PO_4$, 0.15 g/l KCL, 0.37 g/l $MgSO_4$, and 0.15 g/l betaine along with trace elements and a low concentration of 2 g/l xylose as a sole carbon source was prepared for the ALE experiments (Martinez et al., 2007). Agar in 1.5 % concentration was used for a solid medium.

Laboratory Adaptive Evolution Experiments

Four strains of *E. coli* K12, DH5α, BL21, and BW25113 were used in this study. The prepared stock cultures were transferred to LB agar plates and incubated overnight at 37 °C for revival. For adaptation purposes, single colonies from each strain were inoculated into 50ml baffled shake flasks containing 10 ml AM1 + 2 g/l glucose liquid medium and incubated under fully aerobic conditions at 37 °C and 150 RPM to reach the stationary phase. Subsequently, inoculate with OD_{600} of 0.1 from each shake flask were transferred to fresh AM1 + 2 g/l xylose medium. Sub culturing was repeated in three consecutive stages along a control. After each stage, the resulting strains were stored at -80 °C.

Specific growth rate (μ) calculation

The specific growth rate (μ) is defined as the rate of increase in biomass of a cell population per unit concentration of biomass (Bhatia et al., 2015). It is typically expressed in units of h^{-1} and is calculated during the logarithmic growth, where growth reaches its maximum (Dalgaard and Koutsoumanis 2001). Growth curves of the strains were monitored using a spectrophotometer at 600 nanometers. The specific growth rates of each strain were determined from the logarithmic phase of the growth curve and were calculated using the following equation:

$$\mu = \frac{1}{t} \ln \left(\frac{do}{doi}\right)$$

In this equation, ln (do/doi) represents the natural logarithm of the ratio of final OD (do) to the initial OD (doi), where t is the time interval during which growth occurs (Schuler and Marison 2012).

The calculated growth rates were recorded in Microsoft Excel. Linear regression, the equation of the line, and the coefficient of determination were computed using Excel for the obtained data. Growth curve plots for the strains in different passages and specific growth rate calculations were generated using OriginLab software (2018). Two-way ANOVA Sidak's multiple comparisons test was used for the comparison of specific growth rate means between strains. A multiple t-test was conducted to compare the means between the initial culture and the third subculture on xylose, utilizing the Holm-Sidak method with an alpha level set at 0.05. All statistical analyses were done by GraphPad Prism 8.0.2.

Results

Adaptation of E. Coli strains to xylose consumption

Each strain was initially cultured using AM1 + 2 g/l glucose medium, followed by adaptive laboratory evolution (ALE) experiments in AM1 containing 2 g/l xylose medium. The liquid culture, where xylose served as the sole carbon source, was repeated three times using the fresh medium, during which the growth curves of the strains were plotted (Figure 1). The results of our study indicate that DH5α and K12 evolved more rapidly and efficiently for optimal xylose utilization with respectively 57 % and 33 % increase in cell density based on OD_{600} measurement. At the same time, the BW25113 strain showed little change of only 18 %, while BL21 lost its fitness by 26 % during the ALE experiments. It leads to the conclusion that strains within the same species can exhibit different feedback responses to evolutionary pressures. Nonetheless, closely related strains such as DH5α and K12 have demonstrated a similar trend in their fitness improvements when grown on xylose as the exclusive carbon source.

Jung Min Heo et al. (2021) demonstrated that the strains BW25113, BL21 (DE3), MG1655, and strain C consumed xylose at different rates, with *E. coli* BL21 (DE3) exhibiting the slowest xylose consumption rate. This finding was also observed in the

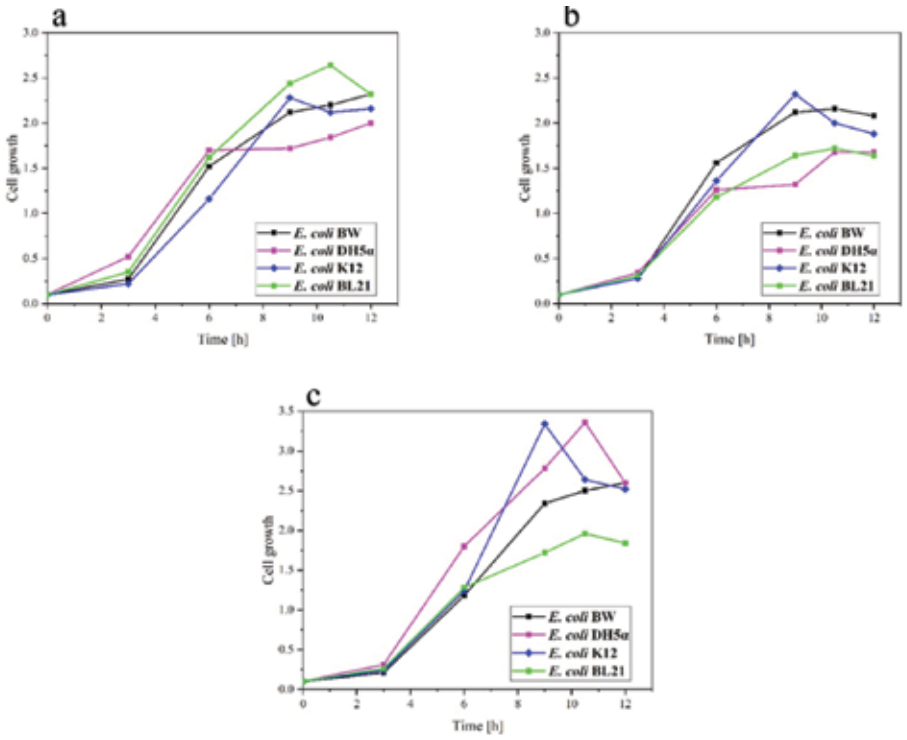

Fig. 1. Growth curves of the studied *E. coli* strains under aerobic conditions at 37 °C and 150 RPM for 12 hours, plotted at a wavelength of 600 nm. Panels a, b, and c represent the first, second, and third passages, respectively. The different *E. coli* strains are depicted in four colors: black for *E. coli* BW25113, purple for *E. coli* DH5α, blue for *E. coli* K12, and green for *E. coli* BL21

present study (Heo et al., 2021). However, in their experiment, only the BL21 (DE3) strain was selected for ALE experiments, whereas in the current study, all four strains were examined. It is important to note that different selective pressures are applied in these two studies.

Analysis of growth rates of strains using a specific growth rate

The microbial growth examination using the specific growth rate (μ) is one of the accurate and essential methods in microbiology (Zhang et al., 2017). As mentioned, the specific growth rate was calculated solely for the logarithmic phase. Four strains were analyzed simultaneously, with each being consecutively subcultured three times. For each of these passages, the corresponding growth rate curves for the selected range were plotted (Figures 2, 3, 4, and 5). The differences in specific growth rates calculated for the strains K12, DH5α, and BW25113 between the third and first cultures were 0.138, 0.376, and 0.046 which is statistically significant at p-values of 0.01, 0.0002, and 0.02, respectively. BL21 strain has shown a small difference of 0.007 which was not statistically significant (Table 1, Figure 6). Furthermore, The Two-way ANOVA test has revealed significant differences (p values = 0.0001) in the mean values (μ) among different strains and subcultures.

In the initial subculture, BL21 exhibited the highest growth rate, with no significant differences observed between K12 and DH5α. However, following adaptation to xylose, DH5α demonstrated an increased growth rate, thereby achieving a significant advantage over K12 and all other strains (Table 2). Additionally, the differences in specific growth rates calculated, as shown in the equations of the lines in Figures 2, 3, 4, and 5, between the third and second cultures were 0.0864, 0.2745, 0.0886, and 0.0644, respectively.

By analyzing the equations of the lines for the three curves, the differences in slope between the first and third passages, and the second and third passages were found to be 0.0583 and 0.0644, respectively

These numbers indicate that the *E. coli* DH5α adapts continuously to xylose consumption at the highest rate. Furthermore, based on the obtained numbers and the analysis of Figure 1, it is evident that the *E. coli* K12 strain also adapts to xylose at a satisfactory rate.

It can also be noted from Figure 1 that the *E.*

Table 1. Multiple comparison t-test between first and third subcultures. Statistical significance at P< 0.05

Strains	P value	Mean of subculture1	Mean of subculture3	Difference	SE of difference
BW	0.013	0.338	0.3845	-0.0465	0.01094
BL21	0.504	0.6434	0.6357	0.0077	0.01051
K12	0.003	0.4036	0.5417	-0.138	0.02238
DH5α	0.00006	0.3936	0.7698	-0.3763	0.02143

coli BW25113 strain demonstrates a strong capacity for xylose consumption; however, this strain did not display any significant alterations throughout the ALE experiments. Conversely, the results for the *E. coli* BL21 strain revealed a lack of adaptation to xylose consumption. Nevertheless, in its baseline condition, this strain showed the capability to utilize xylose under aerobic conditions, similar to the other strains.

By analyzing the equations of the lines for the three curves, the differences in slope between the first and third passages, and the second and third passages were found to be 0.3803 and 0.2745, respectively. These results indicate that this strain adapts to xylose consumption at the highest rate and continuously compared to the other strains

By analyzing the equations of the lines for the three curves, the differences in slope between the first and third passages, and the second and third passages were found to be 0.0491 and 0.0864, respectively. These results indicate that this strain also exhibits relatively good adaptation to xylose consumption under aerobic conditions with a low concentration of xylose.

By analyzing the equations of the lines for the three curves, the differences in slope between the first and third passages, and the second and third passages were found to be -0.0115 and 0.0886, respectively. These results indicate that this strain does not exhibit short-term adaptation to xylose consumption under aerobic conditions with a low concentration of xylose.

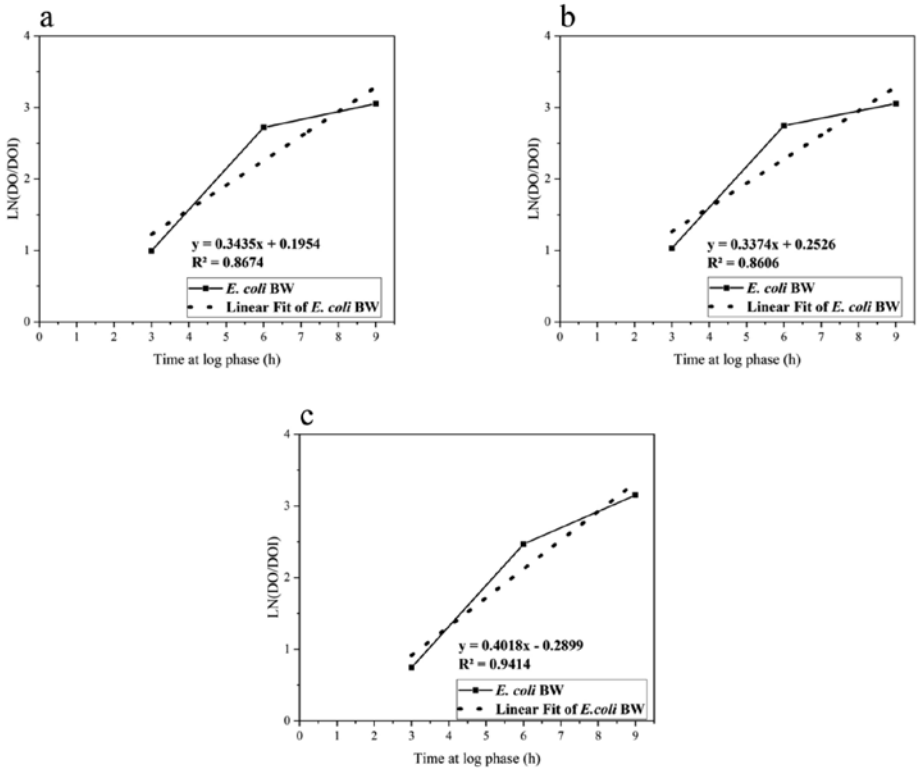

Fig. 2. The specific growth rate for the logarithmic phase (6 hours of the growth curve) of the *E. coli* BW25113 strain is shown as a, b, and c for three passages performed

Table 2. Two-way ANOVA and multiple comparison analysis between strains and subcultures; P < 0.05

Source of Variation	% of total variation	P value	P value summary	
Interaction	24.73	<0.0001	****	
strains	52	<0.0001	****	
subcultures	21.9	<0.0001	****	
Subject	0.8607	0.2322	ns	
Sidak's multiple comparisons test	Mean Diff.	95.00 % CI of diff.	Adjusted P Value	Summary
subculture1				
BW vs. BL21	-0.3054	-0.3572 to -0.2537	<0.0001	****
BW vs. K12	-0.06563	-0.1174 to -0.01391	0.0093	**
BW vs. DH5a	-0.05557	-0.1073 to -0.003841	0.0316	*
BL21 vs. K12	0.2398	0.1881 to 0.2915	<0.0001	****
BL21 vs. DH5a	0.2499	0.1981 to 0.3016	<0.0001	****
K12 vs. DH5a	0.01007	-0.04166 to 0.06179	0.9935	ns
Subculture 3				
BW vs. BL21	-0.2512	-0.3030 to -0.1995	<0.0001	****
BW vs. K12	-0.1572	-0.2089 to -0.1054	<0.0001	****
BW vs. DH5a	-0.3853	-0.4371 to -0.3336	<0.0001	****
BL21 vs. K12	0.09407	0.04234 to 0.1458	0.0003	***
BL21 vs. DH5a	-0.1341	-0.1858 to -0.08237	<0.0001	****
K12 vs. DH5a	-0.2282	-0.2799 to -0.1764	<0.0001	****

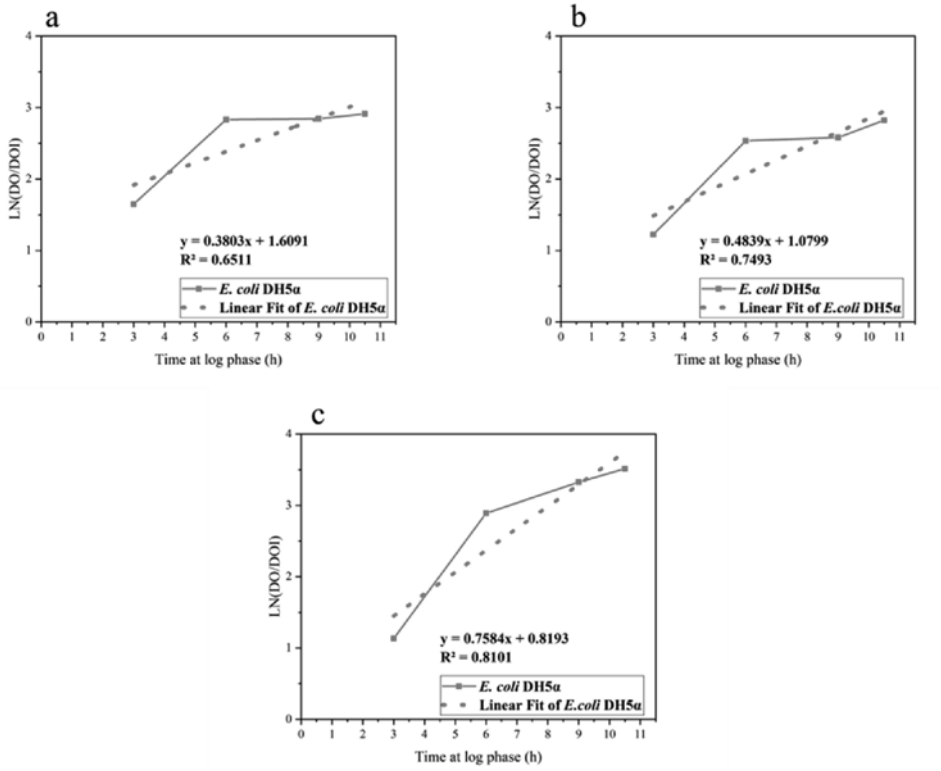

Fig. 3. The specific growth rate for the logarithmic phase (7.5 hours of the growth curve) of the *E. coli* DH5α strain is shown as a, b, and c for three passages performed

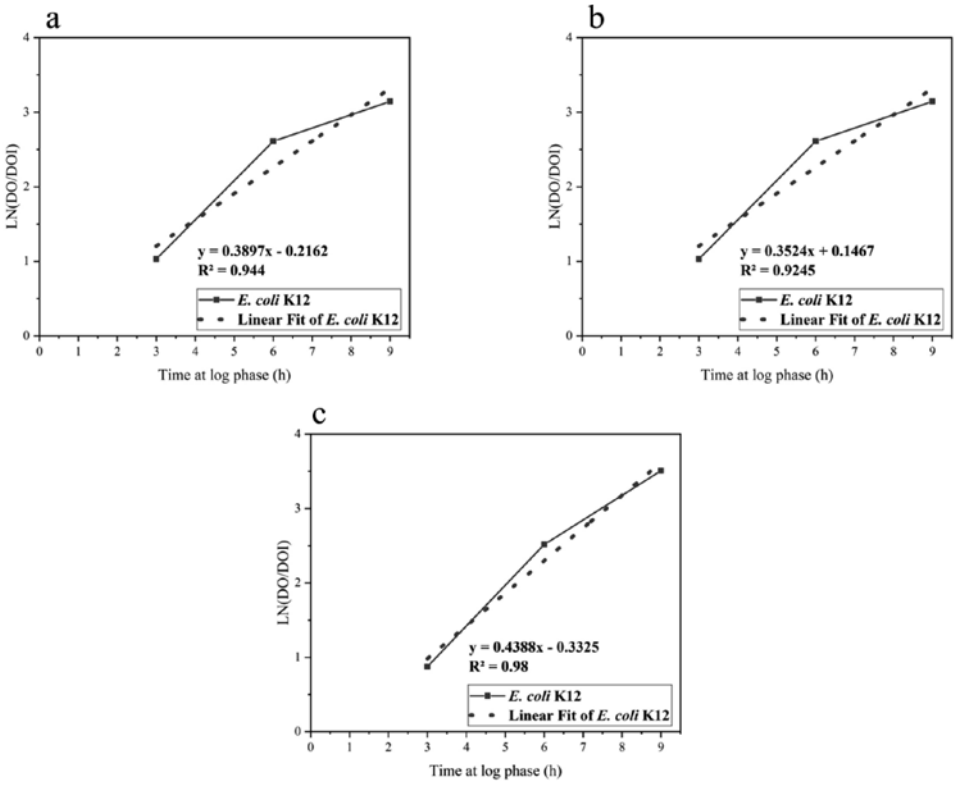

Fig. 4. The specific growth rate for the logarithmic phase (6 hours of the growth curve) of the *E. coli* K12 strain is shown as a, b, and c for three passages performed

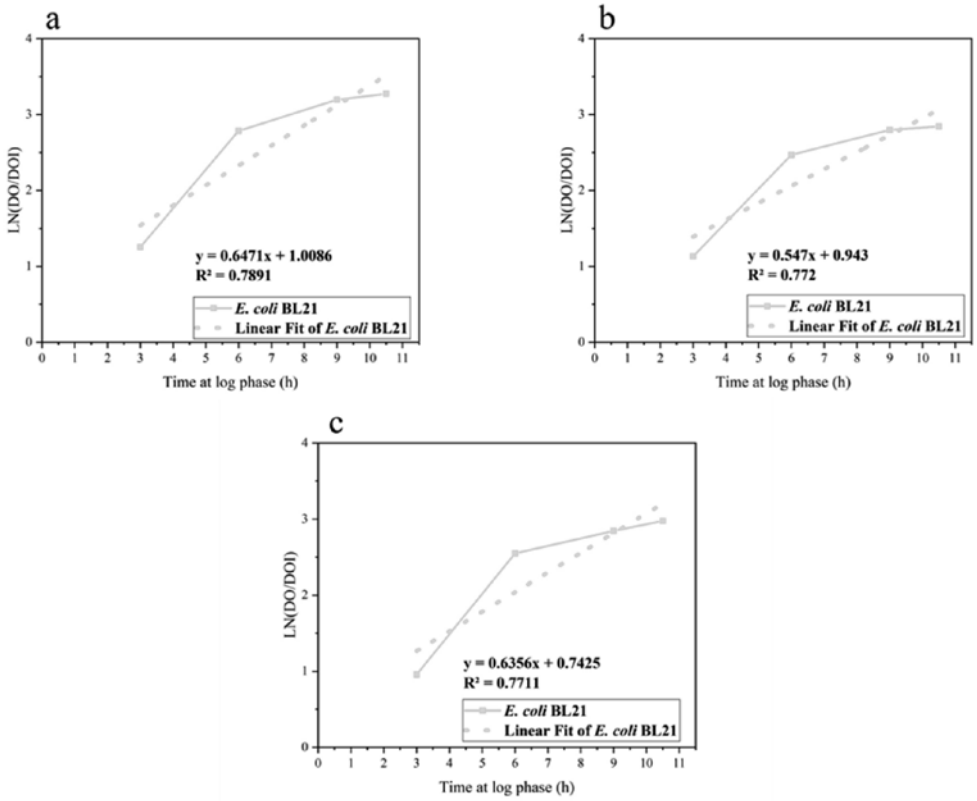

Fig. 5. The specific growth rate for the logarithmic phase (7.5 hours of the growth curve) of the *E. coli* BL21 strain is shown as a, b, and c for three passages performed

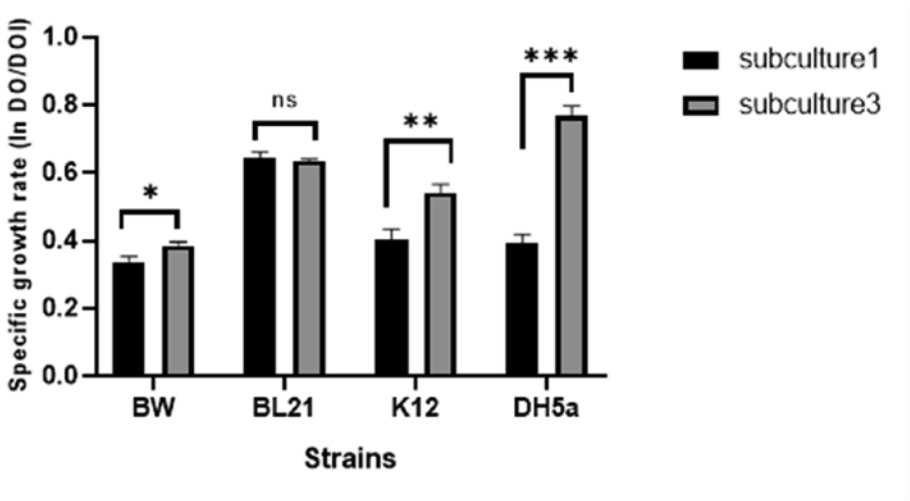

Fig. 6. Comparison of specific growth rates of different *E. coli* strains after two subcultures on AM1 medium containing 2 g/l xylose. A significant increase in the growth rate was observed in BW, K12, and DH5a strains. $p < 0.05$, *; $p < 0.01$, **; p ,0.001, ***

Discussion

The *E. coli* adaptation to growth on xylose is attributed to mutations in various genes in bacteria. In a study, a point mutation was identified in the *gatC* gene, leading to a change from serine to leucine at position 184 of the GatC protein. The deletion of the mutated *gatC* in several strains and the introduction of the mutated *gatC* gene into a wild-type strain confirmed its activity as a xylose transporter, demonstrating that this mutation is responsible for the high xylose consumption phenotype in the strain (Utrilla et al., 2012). Previous studies have revealed that there have been no engineering efforts aimed at enhancing xylose transporters of *E. coli* through any methodology, thereby creating a significant opportunity for researchers. Notably, in the work of Espeso and colleagues (2021), a mutation was introduced in the *xylE* gene, modifying amino acid 33 from glycine (GGT) to valine (GTT). This mutation is likely to increase the transport of xylose by the XylE transporter (Espeso et al., 2021). Another study employed short-term evolutionary engineering using high concentrations of xylose and xylose-glucose, resulting in mutations R121C and P363S in the *xylR* gene, which significantly increased xylose uptake and metabolism in the microorganism (Sievert et al., 2017). Additionally, Yoon et al. (2021) utilized short-term adaptive evolution under anaerobic conditions with low concentrations of xylose and glucose, introducing mutations C91A (Q31K) and C740T (A247V) in the *xylR* gene, which also contributed to enhanced xylose consumption). These studies collectively demonstrate that by altering growth conditions and selective pressures, various genes involved in xylose uptake and metabolism are affected and undergo modifications.

According to the studies by Chuan Ren and colleagues in 2009, the glucose transporter from *Zymomonas mobilis* was transferred

to *E. coli*, and mutations in this transporter were generated and identified using error-prone PCR and random deletion methods, resulting in an improvement of xylose transport by up to 48 %. Furthermore, in the presence of both glucose and xylose, the transport of glucose was only 28 % higher than that of xylose (Ren et al., 2009). the application of random mutagenesis and site-saturation mutagenesis techniques on the Glf transporter, which shares structural and functional similarities with the XylE transporter, led to the generation of mutations that achieved an increase in productivity by approximately tenfold. Additionally, these mutations had an impact on the carbon catabolite repression, allowing xylose to be transported by this transporter in the presence of glucose (Kurgan et al., 2022). These studies collectively demonstrate that by altering growth conditions and selective pressures, various genes involved in xylose uptake and metabolism are affected and undergo modifications.

In conclusion, our experiments adapting four *E. coli* strains for enhanced growth on xylose indicate that *E. coli* K12 and DH5α are the most effective for xylose consumption, making them optimal choices for rapid strain development. In contrast, *E. coli* BL21 demonstrated suboptimal performance in xylose-containing media, rendering it less suitable for quick strain optimization; however, it may be valuable for long-term studies on xylose adaptation. This research is aligned with recent advancements in the bioconversion of xylose into useful chemicals, where various wild-type and engineered microbial strains have been developed to efficiently produce compounds such as ethanol, 1,4-butanediol (BDO), and xylitol (Francois, Alkim, and Morin 2020). These findings underscore xylose's potential as a renewable carbon source for synthesizing high-value chemicals and biofuels, promoting sustainable bioprocessing methods in lignocelluloses' biomass utilization. The regulatory mechanisms governing xylose metabolism in *E. coli* further enhance its applicability in bioconversion processes, positioning it as a key player in the development of efficient fermentation technologies.

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