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Yong-Shui Tan
Tianjin University
Li Wang
Tianjin University
Ying-Ying Wang
Southeast University
Qi-En He
Tianjin University
Zhen Zhu
Southeast University
Kai Song
Tianjin University
Zhi-Hua Liu
Tianjin University
Bing-Zhi Li (bzli@tju.edu.cn)
Tianjin University
Ying-Jin Yuan
Tianjin University

Research

Keywords: Xylose consumption memory, Protein acetylation, Xylose stress, Acetylation-related enzymes, Saccharomyces cerevisiae, H4K5, HPA2

Posted Date: September 20th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-910475/v1

Version of Record: A version of this preprint was published at Biotechnology for Biofuels on December 1st, 2021. See the published version at https://doi.org/10.1186/s13068-021-02090-x.

Protein acetylation regulates xylose metabolism during adaptation of *Saccharomyces cerevisiae*

Yong-Shui Tan [a,b], Li Wang [a,b], Ying-Ying Wang [c], Qi-En He [d], Zhen Zhu [c], Kai Song [d], Zhi-Hua Liu [a,b], Bing-Zhi Li [a,b] *, Ying-Jin Yuan [a,b]

[a] Frontiers Science Center for Synthetic Biology and Key Laboratory of Systems Bioengineering (Ministry of Education), School of Chemical Engineering and Technology, Tianjin University, Tianjin, 300072, PR China

[b] Synthetic Biology Research Platform, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin University, Tianjin, 300072, PR China

[c] Key Laboratory of MEMS of Ministry of Education, Southeast University, Nanjing, 210096, PR. China

[d] School of Chemical Engineering and Technology, Tianjin University, Tianjin, 300072, PR China

Yong-Shui Tan, E-mail: ystan1001@163.com

Li Wang, E-mail: Lilywang_NK@163.com

Ying-Ying Wang, E-mail: yy_wang1994@seu.edu.cn

Qi-En He, E-mail: heqien777@tju.edu.cn

Zhen Zhu, E-mail: zhuzhen@seu.edu.cn

Kai Song, E-mail: ksong@tju.edu.cn

Zhi-Hua Liu, E-mail: zhliu@tju.edu.cn

Ying-Jin Yuan, E-mail: yjyuan@tju.edu.cn

*Corresponding author: Prof. Bing-Zhi Li, bzli@tju.edu.cn

[†]Equal contribution.

Abstract

Background: Lignocellulosic biomass upgrading has become a promising alternative route to produce transportation fuels in response to energy security and environmental concerns. As the second most abundant polysaccharide in nature, hemicellulose mainly containing xylose is an important carbon source that can be used for the bioconversion to fuels and chemicals. However, the adaptation phenomena could appear and influence the bioconversion performance of xylose when *Saccharomyces cerevisiae* strain was transferred from the glucose to the xylose environment. Therefore, it is crucial to elucidate the mechanism of this adaptation phenomena, which can guide the strategy exploration to improve the efficiency of xylose utilization.

Results: In this study, xylose-utilizing strains had been constructed to effectively consume xylose. It is found that the second incubation of yYST218 strain in synthetic complete-xylose medium resulted in a 1.24-fold increase in xylose consumption ability as compared with the first incubation in synthetic complete-xylose medium. The results clearly showed that growing *S. cerevisiae* again in synthetic complete-xylose medium can significantly reduce the stagnation time and thus achieved a faster growth rate, by comparing the growth status of the strain in synthetic complete-xylose medium for the first and second time at the single-cell level through Microfluidic technology. Although these xylose-utilizing strains possessed different xylose metabolism pathways, they exhibited the “transient memory” phenomenon of xylose metabolism after changing the culture environment to synthetic complete-xylose medium, which named ‘xylose consumption memory (XCM)’ of *S. cerevisiae* in this study. According to the identification of protein acetylation, partial least squares analysis and the confirmatory test had verified that H4K5Ac affected the state of “XCM” in *S. cerevisiae*. Knockout of the acetylase-encoding genes

GCN5 and *HPA2* enhanced the "XCM" of the strain. Protein acetylation analysis suggested that xylose induced perturbation in *S. cerevisiae* stimulated the rapid adaptation of strains to xylose environment by regulating the level of acetylation.

Conclusions: All these results indicated protein acetylation modification is an important aspect that protein acetylation regulated the state of "XCM" in *S. cerevisiae* and thus determine the environmental adaptation of *S. cerevisiae*. Systematically exploiting the regulation approach of protein acetylation in *S. cerevisiae* could provide valuable insights into the adaptation phenomena of microorganisms in complex industrial environments.

Keywords: Xylose consumption memory; Protein acetylation; Xylose stress; Acetylation-related enzymes; *Saccharomyces cerevisiae*; H4K5; *HPA2*

Background

High dependence on fuel and gradual exhaustion of fossil energy has generated the need for an alternative renewable energy source. Biomass is an important renewable source obtained from energy crops, aquatic plants, forest biomass, and agricultural residues. Biofuels from biomass refinery have attracted more attention and have been considered to be the most promising alternative [1-3]. As the first generation bioethanol produced from food starch affected the availability of food to humans and consumed arable land [4], the second generation bioethanol had been developed using non-food lignocellulosic biomass to address the need of liquid fuel for vehicles [5-7]. Production of lignocellulosic bioethanol is mainly dependent on the constitutes of lignocellulosic biomass [8-10].The pentose and hexose polymerized carbohydrates in lignocellulosic biomass are the main compositions which can be converted into fermentable glucose and xylose, respectively, and then second-generation bioethanol. Xylose, as a kind of

pentose in lignocellulosic biomass, has the potential to be used by microorganisms to produce bioethanol [11, 12]. Besides, to reduce the cost of bioethanol, it is also necessary to maximize the utilization of these compositions especially completely convert the xylose. Many microorganisms have been chosen as suitable hosts for xylose conversion and various genetic engineered tools and approaches have been exploited to improve the conversion efficiency of xylose by these hosts. Among these microorganisms, *S. cerevisiae* has been favored not only because of its excellent tolerance against harsh industrial conditions, but also due to the sample genetic engineering tools available and the strong potential of xylose metabolism [13-15].

Generally, the strain cells can store the information about current environment when encountering environmental changes, which allows the strain responds quickly to utilize nutrients when returning to original environment again [16, 17]. In a study of galactose memory, the memory-induced *GAL* genes (*GAL1*, *GAL2*, *GAL7*, and *GAL10*) in *S. cerevisiae* can be rapidly activated within 7 generations of division when the strain was cultured on galactose medium [18-20]. The expression levels of the corresponding galactose metabolizing enzymes in *S. cerevisiae* changed correspondingly when the concentrations of glucose and galactose were transformed [21]. Heterokaryons formed by mating galactose-induced and un-induced S. cerevisiae cells were found to showed a memory phenotype when the placement of un-induced cell heterokaryons were placed in the galactose-induced -cell cytoplasm. This reveals that the cytoplasmic factor of in the galactose- induced cells contain substances that can induce this memory [19]. Since microorganisms can store those memories in the form of molecular interactions, this phenomenon generally called “transient memory” [22]. Epigenetic regulation plays a critical role in allowing organisms to adapt to their environment. Many previous studies have revealed that there is close

89 link between epigenetic and microorganism memory. It was previously found that after a period of
90 adaptation in an environment lacking inositol, newly germinated progeny *S. cerevisiae* cells were
91 able to rapidly maintain the homeostasis of the *INO1* gene [18, 23-25], and the regulation of the
92 *INO1* gene is in turn influenced by multiple factors such as the *SFL1* transcription factor, H2A.Z,
93 and methylation of histone H3 [26, 27]. Sudden environmental changes may cause microbe
94 entering protective state, exhibiting growth stagnation and slow growth [28]. Thus, epigenetic
95 modification causes the regulation at the genetic level of microorganisms to generate "transient
96 memory" in the metabolic network [29-31].

97 Glucose is an ideal substrate for ethanol production by *S. cerevisiae*, but xylose generally can
98 not be effectively consumed by wile-type *S. cerevisiae*. The engineering of xylose metabolism
99 pathway in *S. cerevisiae* has been investigated and the bioconversion of xylose in lignocellulosic
100 hydrolysates has been achieved by xylose-utilizing *S. cerevisiae* in previous studies. The next
101 major question what we need to pay more attention is the behaviors of *S. cerevisiae* when *S.*
102 *cerevisiae* is cultured on glucose and xylose in lignocellulose hydrolysates. As *S. cerevisiae* will
103 switch from the culture environment with glucose to xylose during the fermentation progress, it
104 could greatly affect the growth state and thus fermentation performance of the strain due to rapidly
105 changing the fermentation environment. Previous studies confirmed that the environmental
106 alterations caused epigenetic changes and stabilized the survival of cellular progeny. However, it
107 is still unknown whether the epigenetic changes can alter protein acetylation in eukaryotic
108 microorganisms and whether it can enable *S. cerevisiae* to adapt rapidly to the xylose culture
109 environment and pass this ability to its progeny. Therefore, detailed information about the
110 behaviors of *S. cerevisiae* on the culture change between glucose and xylose needs to be illustrated,

which should provide guidance for improving the xylose utilization performance.

The present study aims to reveal the "transient memory" of *S. cerevisiae* induced by switching culture between synthetic complete-glucose medium (SG) and synthetic complete-xylose medium (SX) and systematically elucidate the molecular mechanism of this phenomena during the culture switching process. The experiments were first designed by switching culture between SG and SX environment to induce the "transient memory" of *S. cerevisiae.* The protein acetylation analysis of xylose-induced *S. cerevisiae* had been employed to prospect the acetylation-related genes related to the xylose memory phenomena. The least-squares statistical analysis of protein acetylation data had been used to identify several key acetylation locations. The mutation of acetylation site was then conducted to validate the effects of specific acetylation site on xylose memory phenomenon. These results could be helpful to get insights into the adaptation phenomena of *S. cerevisiae* in new environments, guiding the fermentation process design for improving xylose utilization performance.

Results

Microfluidic culture validated the "XCM" of *S. cerevisiae*

To obtain the xylose-utilizing *S. cerevisiae* strains for xylose adaptation study, the xylose reductase metabolizing the yYST201 strain (MATα, his3Δ1, leu2Δ0, lys2Δ0, and ura3Δ0) was constructed using BY4742 strain as the chassis, and the yYST218 strain (MATalpha, leu2-3, leu2-112, ura3-52, and trp1-298 can1 cyn1 gal+) was constructed using L2612 strain as the chassis [32, 33]. The reported xylose utilization the SQ-2(yYST009) strain in our lab was also employed in the present study [34] (Fig. 1A).

These strains were first cultured in SG and then transferred to SX. After culturing for 24

hours, these strain cells were transferred to SX again, and cultured under SX for another 24 hours. Results showed that each chassis strain with specific metabolic pathway has individual xylose consumption and cell growth rate, indicating the different cell behaviors among these strains. Previous studied reported that *S. cerevisiae* can adapt quickly to the changed culture environment under the regulation of the stress-related genes, such as the high-salt environment [35]. As wild-type *S. cerevisiae* will preferentially consume glucose and can not metabolize xylose, xylose could be as the changed culture environment like an "induction condition". Interestingly, the xylose consumption rate of the yYST218 strain in SX for 12 h for the first time was 0.108 g/h, and the xylose consumption rate for 12 h after the second transfer to SX was 0.243 g/h, and the xylose consumption rate increased by 1.24 times. It was also found that the xylose consumption rate of the yYST009 strain was increased by 0.48 times, and the xylose consumption rate of the yYST201 strain was increased by 0.63 times. After transferring the strain cells to SX for the second time, it was observed that all the strains were able to consume xylose faster compared with that for the first inoculation. Results suggested that these xylose-utilizing strains with different xylose metabolism pathways exhibited the memory phenomenon of xylose metabolism after changing the culture environment to SX.

Microfluidic technology could be an effective tool to observe the phenotypic changes of microbes by changing environment [36]. To validate whether *S. cerevisiae* can quickly adapt to the changing environment, a microfluidic device was employed to cultivate an ever-growing microbial population in the micro-growth chamber. Using this microfluidic technology, the memory phenomena could be evaluated by capturing and cultivating *S. cerevisiae* cells. During the culture, the budding daughter yeast cells would detach from the bottom hole of the micro-

155 growth chamber by applying fluid pressure from the upstream of the cells. This helped us to
156 determine each cell's growth status on the budding station (Fig. 2A). The different xylose-utilizing
157 strains were first activated in SG in test tubes, respectively. The different strain cells were then
158 injected into the chip, respectively, so that enough capture structures can capture a single cell. At
159 the same time, SG was injected into the main channel, and the medium flowing in the main
160 channel was switched from SG to SX after 4 h culture (Fig. 2B). When the environment changes
161 for the first time, all strains immediately entered a growth arrest state, suggesting that cell stopped
162 dividing at this state. As the different strains possessed the capacity of xylose adaptation, the
163 duration of the stasis phase varied from 2.5 h to 5 h. After the stasis phase, the strain cells will
164 enter the recovery phase and gradually return to the normal state of division. A similar stagnation
165 period of *S. cerevisiae* was observed when switched to SX from SG. After culturing in SX for 10 h
166 the flowing medium in the main channel was switched to SG again. This environmental change
167 did not cause the stagnation of cell growth, and the cells were able to switch to SG growth state
168 without a trace. Finally, after 4 h culture in SG, the flowing medium in the main channel was
169 switched to SX again. Interestingly, the second SX culture did not cause a significant metabolic
170 burden on the cells, and the cell growth was restored in a shorter time compared with the first SX
171 culture (Fig. 2C). According to the first and second delay period, the stagnation rate is obtained,
172 and the stagnation rate of yYST24, yYST31, yYST48, yYST54, yYST55, and yYST201 are
173 calculated to be 1.52, 2.07, 1.79, 1.96, 2.31, 1.58, respectively. Overall, comparing the strain
174 growth state on SX for the first and the second time, results clearly suggested that *S. cerevisiae*
175 culturing in SX again can significantly shorten the stagnation time to enable a faster grow rate
176 than that for the first SX culture (Fig. 2D). The microfluidic culture had thus confirmed the

behaviors of the memory for the xylose-utilizing *S. cerevisiae.* Through fermentation experiments and microfluidic experiments, it was found that the consumption ability of xylose by all *S. cerevisiae* after the xylose culture was accelerated, just like *S. cerevisiae* remembered xylose, which named 'xylose consumption memory (XCM)' of *S. cerevisiae* in this study.

Xylose-stress induced protein acetylation of *S. cerevisiae*

The environmental stress will cause the changes of epigenetic regulation in plants and animals, and these changes could be inherited for several generations [37]. For example, previous studies found that the genome methylation on the growth of plants near the Chernobyl Reactor was changed under radiation stress tolerance conditions, which increased the stability of the genome [38, 39]. Generally, the ubiquitous epigenetic phenomenon involves all levels of gene regulation, and epigenetic modification controls various states of cell growth. As aforementioned, the cultivation of *S. cerevisiae* in an environment with the switch of xylose and glucose can be seen as "environmental pressure". This stress-induced environment was established for *S. cerevisiae*, which could be used to evaluate the epigenetic phenomenon of the strain cells.

Protein acetylation is an important epigenetic modification, which is helpful to discover relationships between epigenetic modification and "XCM" phenomena. To analyze the potential epigenetic modification of *S. cerevisiae*, the culture strategy was furth designed to obtain the protein acetylation information. In details, the yYST218 strain with xylose-utilizing ability was continuously subcultured in SG for 9 d (D9) and make the cells fully adapt to the growth environment in SG. The yYST218 strain was then transferred to SX for continuous subculture for 6 d (D9X6) and make the strain fully adapt to the culture environment of SX, induces the ability of the bacteria to produce memory for xylose. Finally, the yYST218 strain was transferred to SG

and cultured for 9 d (D9X6D9), so that it will completely loss the memory to xylose metabolism ability (Fig. 3A). The potential protein acetylation of these cells had further been conducted using tandem mass tags (TMT) labeling and Kac affinity enrichment followed by high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The quantitative lysine acetylome analysis was performed in pair of *S. cerevisiae* strains. Altogether, 871 lysine acetylation sites in 420 protein groups had been identified, among which 841 lysine acetylation sites in 403 proteins had been quantified. The fold-change cutoff was set as proteins with quantitative ratios above 1.2 or below 0.83 were deemed significant. Among these quantified sites, 41 lysine acetylation sites were up-regulated while 47 lysine acetylation sites were down-regulated in D9X6 vs D9. 3 lysine acetylation sites were up-regulated while 36 lysine acetylation sites were down-regulated in D9X6D9 vs D9. 23 lysine acetylation sites were up-regulated while 51 lysine acetylation sites were down-regulated in D9X6D9 vs D9X6(Fig. 3B).

These results suggested that the behaviors of xylose-utilizing *S. cerevisiae* induced by the switch of SG and SX medium could be closely related to protein acetylation modification. However, different protein acetylation sites could have different correlations with the xylose utilization and thus the behaviors of xylose-utilizing *S. cerevisiae*. Therefore, it is very important to predict key protein acetylation sites by identifying the potential characteristic sites and their inferences on related regulatory network, which could be facilitate to understand the molecular mechanism of “XCM”. A new partial least squares deep regression method was employed to analyze the protein acetylation. To compare xylose D9, D9X6 and D9X6D9, the prediction model of acetylation sites had been further optimized. Three culture scenarios, including D9, D9X6 and D9X6D9, were transformed into 3 binary classification problems (6 samples, 3 positive and 3

negative). The most judgmental sites had been identified using the partial least squares depth regression algorithm. After removing the sites with missing values, 324 sites (variables) remained in the acetylation group. Combined with the variable importance index (VIP), all variables had been arranged in descending order of importance (Additional file 3). The larger the VIP value, the more important the acetylation site is. In the comparison of D9X6 vs D9, it is found that the VIP values of H4K5, H4K8, H3K27, and H4K16 are 1.3136, 1.3135, 1.3122, and 1.3111 respectively, which are larger than the VIP values of other acetylation sites. In the comparison of D9X6 vs D9X6D9, it is found that the VIP values of H4K5, H4K8, H3K27, and RPL37AK51 are 1.3529, 1.3527, 1.3514, and 1.3511 respectively, which are larger than the VIP values of other acetylation sites. The result indicated that the acetylation sites including H4K5Ac, H4K8Ac, H3K27Ac may play an important role in "XCM" (Fig. 3C).

H4K5 and H4K8 acetylation regulated the "XCM"

Several key protein acetylation sites had been identified in the xylose-utilizing *S. cerevisiae* by aforementioned analysis. It is necessary to further evaluate the specific correlations between these sites and "XCM". Two acetylation sites, H4K5 and H4K8, had been selected according to VIP indicators. The lysines at H4K5 and H4K8 positions were mutated to arginine, by which the ability of acetylation was eliminated. The corresponding mutate the yYST210 (H4K5R) and yYST211 (H4K8R) strains were obtained. To evaluate the "XCM" behavior of these mutation, the strains were subcultured in SX for 8 d. After that, these strains were transferred to SG and cultured for 0, 8, 12, 16, and 20 d, respectively. Finally, these strains after culture in SG were transferred to SX to evaluate the ability of "XCM". For these experiments, xylose consumption was analyzed at the same culture time (Fig. 4A). Interestingly, results clearly showed that the loss of acetylation at

H4K5 site significantly accelerated xylose consumption ability by the mutate strain, while the loss of acetylation at H4K8 site slightly changed the xylose consumption ability during the culture process. Moreover, the xylose consumption ability of the yYST211 strain (H4K8R) and the control strain (yYST218) were basically stable and similar after 8 d continuous culture in SG. After 20 d consecutive culture in SG, the xylose consumption ability of these three strains did not change significantly (Fig. 4B-4F). The mutate strains were constructed and induced to possess the ability memory of "XCM" and then to gradually lose "XCM" ability by cultivating them in SG. These strains lost "XCM" were transferred to SX and cultured for 58 hours. The xylose consumption obtained was compared to obtain the xylose forgetting value. The xylose forgetting value of various strains had been calculated based on equation S5 in methods. Through calculations, it is found that the forgetting values of Control, H4K5 and H4K8 are 3.708, 2.504, and 3.373 respectively, and the forgetting values of Control and H4K5 have changed significantly. Interestingly, the loss of acetylation at H4K5 and H4K8 both promoted the xylose consumption ability, while the loss of acetylation at H4K5 also decreased the forgetting speed of "XCM" in *S. cerevisiae* (Fig. 4G). Through this result, we found that changes in the function of a single acetylation site will affect the ability of *S. cerevisiae* to consume xylose.

Acetylation-related enzymes enhanced "XCM"

Aforementioned results suggested that the acetylation modification of a single site would impact the "XCM", however, how global acetylation changes influence the "XCM" was still unknown. The impact of acetylation-related enzymes on "XCM" were thus further investigated. Representative acetylases (*ELP3*, *GCN5*, *SAS3*, and *HPA2*) and four deacetylases (*HDA1*, *HOS2*, *HST1*, and *RPD3*) were selected as they are closely related to the acetylation[40]. These 8 genes

were knocked out individually to evaluate the effects of the deletion of these enzymes on "XCM", respectively. In details, these strains were cultured in SG for 48 h, and then transferred to SX for 24 h. Xylose concentration was measured at the same culture time. These strains had a preliminary memory of xylose metabolism after 24 h culture on SX, and then transferred them to SX (Fig. 5A). Through the above process, the two states that did not produce xylose consumption and the memory of xylose consumption have been produced are compared, and the changes in xylose consumption ability of the strain are analyzed (Fig. 5B-5I). Results showed that the knockout of these genes affected "XCM" at varying degrees. It is interesting to find that knockout of these genes reduced the rate of xylose consumption ability by *S. cerevisiae*, but the behaviors of "XCM" depended on these genes. The xylose memory value of various strains had been calculated based on equation S6 in methods. Results showed that the memory value of the yYST218 of control strain was 1.16. After knocking out the acetylase *HOS2*, the memory value of the yYST246 strain increased to 1.49. Knock-out of acetylase *GCN5* and *HPA2* significantly increased the memory value of the yYST250 and yYST251 strains to 2.06 and 3.00, respectively. Knock-out of other acetylation-related enzymes had no significant effect on the memory value of xylose (Fig. 5J). All these results suggested the representative acetylases (*GCN5*, *HPA2*) were key acetylation-related enzymes to enhance the "XCM" ability of *S. cerevisiae* as compared with other acetylases and deacetylases selected in the present study.

Discussion

The mechanism of the cellular memory phenomenon could involve in several aspects, such as specific protein molecules and epigenetic modifications of proteins[23, 24, 26, 27]. In the present study, protein acetylation modification of *S. cerevisiae* has been investigated to evaluate their

correlation with “XCM”. Two xylose utilization states of strains, that is, no memory of xylose consumption and memory of xylose consumption, had been induced with or without xylose stress. The observation of the growth status of single cells by a microfluidic device confirmed that the strain cells still maintained the “XCM” state for multiple generations after leaving the xylose environment. However, this “XCM” did not exist stably as it is deeply affected by protein post-translational modifications and can be passed on to future generations to function. This established approach could be used as a general strategy for the study of “transient memory” phenomena in other organisms or under other stressful environments. The similar memory phenomena of *S. cerevisiae* on the galactose system had been observed, suggesting that the memory phenomena of *S. cerevisiae* can be induced under specific stress conditions [41, 42]. Post-translational modification of proteins is a key regulatory mechanism in the strain cells to fine-tune protein function and adapt the stress conditions. The lysine acetylation generally occurs on thousands of proteins in various cellular metabolic processes and plays an important role in metabolic regulation [43].

The regulation of xylose by acetylation modification had not been fully revealed in *S. cerevisiae*. Using the partial least squares deep regression method, the protein acetylation identification facilitates the discovery of the key acetylation site (H4K5Ac) in *S. cerevisiae* after the conversion from SG to SX. When switching the strain from SG to SX, the acetylation level of H4K5 had significantly changed, which was supported by previous studies as glucose affects histone acetylation [44]. The variation of culture environment may affect the production of acetyl-CoA and the growth state of cells, thereby affecting the acetylation of histone and the speed of cell proliferation and division [45-47]. The acetylation capacity of H4K5 in *S. cerevisiae* was

309 eliminated by mutating H4K5 to a constitutive deacetylation state (R), which enhanced the xylose
310 depletion capacity and promoted the "XCM" ability. H4 is a subunit of histone, the main protein
311 component of chromatin [48-50]. Together with DNA fragments, histones constitute the basic
312 structure of the genome [51]. Their specific locations make histone modifications important in
313 transcriptional regulation, DNA repair, chromatin cohesion, and environmental adaptation [52]. It
314 is possible that the changes in acetylation capacity of the H4K5 site alters the charge state of the
315 modified disability, interfering with the histidine-histone or histidine-DNA electrostatic
316 interactions and thus leading to a shift in chromatin state to cause a series of regulatory disorders
317 [52]. Since xylose metabolism affects the center of cellular energy metabolism via the pentose
318 phosphate pathway and acetyl coenzyme A is closely related to energy metabolism [53], other
319 acetylation sites may affect the growth state of the cells in the same way.
320 The information about post-translational modifications of H4K5Ac on "XCM" in xylose
321 provided good models to explore the regulation and function of histone acetylation site. However,
322 other possible effects, such as the regulation of acetylases and deacetylases, could not be
323 discharged. Post-translational modifications of histones are essential for the regulation of gene
324 expression in eukaryotes. In our study of the memory capacity of the bacterium for xylose
325 depletion, we found that two genes associated with acetylation (*GCN5* and *HPA2*) knocked out
326 individually affected the memory capacity of the bacterium for xylose depletion, resulting in an
327 increase in the memory value of the *S. cerevisiae*. *GCN5* is a histone acetyltransferase that can
328 catalyze the post-translational modification of multiple lysine sites of histone H3 by transferring
329 acetyl groups to the free amino groups of lysine residues [54]. *GCN5* has a direct correlation with
330 cell growth, in vivo transcription, and in vivo *GCN5*-dependent histone acetylation at the *HIS3*

promoter [55, 56]. *HPA2* is a *S. cerevisiae* protein, a member of the GNAT superfamily, which was tested in vitro and can acetylate histones H3K14, H4K5 and H4K12 [57]. In contrast, losing H4K5 acetylation function in *S. cerevisiae* slowed down the forgetting of "XCM" in *S. cerevisiae*, and knocking out the *HPA2* gene significantly improved the "XCM" in *S. cerevisiae*. These results suggested that H4K5Ac and *HPA2* played an important role in "XCM" of *S. cerevisiae*. There is a reciprocal regulation between cell metabolism and epigenetic modifications. The cellular metabolism influenced the histone modifications, while the alteration of epigenetic modifications on metabolic genes can in turn regulate the expression of metabolic genes. This relationship could provide a more rapid and flexible way for cells to adapt to the changes of the culture environment. Overall, the lysine acetylation could be critical to regulate the "XCM" of *S. cerevisiae* under a xylose-stress condition. The molecular mechanism analysis of the "XCM" could provide a new strategy for the adaptation of *S. cerevisiae* to the complex conditions, such as nitrogen source alteration and inhibitor stress.

Conclusions

The "XCM" in *S. cerevisiae* have been induced with a switch of culture modes and the molecular mechanisms associated with "XCM" had been systematically elucidated from a protein acetylation perspective. Protein acetylation perturbated and regulated multigene pathways, which in turn affected the "XCM" of *S. cerevisiae*. The multiple technologies had been employed to identify the acetylation sites and alter the acetylation modifying related enzymes, and the results confirmed that acetylation modifications significantly affected the ability of "XCM" in *S. cerevisiae*. A systematic exploration of the regulation of protein acetylation in *S. cerevisiae* provides valuable insights into the regulation of microbial adaptation to changes in complex

industrial environments.

Methods

Strains and plasmids

Strains used in this study are summarized in Additional file 1: Table S1. All primers were synthesized by Genewiz (China) and listed in Additional file 1: Table S2. *S. cerevisiae* L2612 (MATa, leu2-3, leu2-112, ura3-52, and trp1-298 can1 cyn1 gal+), BY4742 (MATα, his3Δ1, leu2Δ0, lys2Δ0, and ura3Δ0), syn II, III, VI & IX (MATα, his3Δ1, leu2Δ0, lys2Δ0, and ura3Δ0) were used for constructing recombinant strains. *E. coli* Top10 (purchased from Beijing Biomedical Co., Ltd.) was used for gene cloning and plasmid construction. The required promoters and terminators were amplified from *S. cerevisiae* (S288C), and the genes were synthesized by GenScript China Inc. and then assembled using Vazyme' ClonExpress MultiS One Step Cloning Kit (C113).

Media and culture condition

S. cerevisiae strains were cultivated in a liquid SG medium (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose, 0.1 g/L leucine, 0.02 g/L histidine, 0.02 g/L uracil and 0.02 g/L tryptophan)，SX medium (6.7 g/L yeast nitrogen base without amino acids, 20 g/L xylose, 0.1 g/L leucine, 0.02 g/L histidine, 0.02 g/L uracil and 0.02 g/L tryptophan), synthetic complete medium without uracil (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose, 0.1 g/L leucine, 0.02 g/L histidine, and 0.02 g/L tryptophan). SG medium and SX medium were filtered through a 0.2-μm filter and F127 were added to make a final concentration of 0.5% before injection into the microfluidic system. 100 mg/L ampicillin was added into the Luria–Bertani medium (10 g/L peptones, 5 g/L yeast extract, and 5 g/L sodium chloride) used to cultivate *E. coli*.

Fermentation experiments

The effect of induction time of the *S. cerevisiae* strain in SX medium or SG medium on xylose consumption ability was comparatively analyzed. Fermentations were performed in 250-mL triangular flasks at 30 °C, 200 rpm, and a volume of 100 mL. High-throughput fermentation assays were performed using 96-well plates at 30 °C, 900 rpm, and a volume of 230 μL with a medium of choice (SG or SX). Samples were filtered through 0.2-μm filters before injection into the high-performance liquid chromatography (HPLC) system. Xylose concentration in the fermentation medium was analyzed by HPLC (Waters e2695/2414) using an Aminex HPX-87H ion-exchange column (Bio-Rad, Hercules, USA). The mobile phase was 0.5 mM H_2SO_4 at a flow rate of 0.6 mL/min and the column temperature was 65°C.

Microfluidic culturing and testing

A microfluidic channel integrated with YRot traps was employed in this study [58]. The microfluidic channel has an inlet for the injection of cell suspension and medium, a cell-trap array for single-cell culturing, and an outlet for waste collection. The trap comprises two "L-shaped" pillars, together forming a wide upstream opening for holding cells and an orifice downstream for the dissection of mature daughter cells.

The microfluidic channel was fabricated by the standard polydimethylsiloxane (PDMS)-based soft-lithography process. First, a SU-8 (SU-8 3010, MicroChem Co., USA) master mold was patterned onto a 4-inch silicon wafer using photolithography, followed by silanization with trichloro (1H,1H,2H,2H-perfluorooctyl) silane (Sigma-Aldrich Co., USA) in the vapor phase. Subsequently, the mixture of PDMS oligomer and cross-linking polymer (Sylgard 184, Dow Corning Co., USA) with a weight ratio of 10:1 was poured onto the master mold and was then

degassed by vacuum pumping and cured by baking at 90 °C for 2 h. The PDMS replicas were then carefully peeled off from the master mold, cut into pieces, and holes were punched for the inlet and outlet. The PDMS sheet was irreversibly bonded onto a glass slide by the treatment with oxygen plasma.

In this experiment, the microchannel was first sterilized using 75% ethanol injected from the inlet and baked at 100 °C overnight for residual liquid evaporation. The cell suspension was then injected into the channel at a flow rate of 5 μL/min for 5 min, and then the inlet was switched for fresh SG medium for long-term culturing at a flow rate of 10 μL/min. During the experiments, the microfluidic chip was clamped by a customized holder, with its inlet and outlet connected to a glass syringe and waste collection, respectively. The whole system was together kept on an inverted confocal microscope (FV3000, Olympus Co., Japan) and then imaged using a 20× objective lens (UCPLFLN, 0.7 NA, Olympus Co., Japan, correction ring adjusted to 0.5 mM). Bright-field images were automatically scanned at intervals of 10 min using a software workflow (FV31S-SW, Olympus Co., Japan). The Z-axis Drift Compensation system (IX3-ZDC2, Olympus Co., Japan) allowed the sharp focusing of samples throughout the imaging period.

Protein acetylation analysis of *S. cerevisiae* strains

For the preparation of the yYST218 strain, first it was cultured in the SG medium for 9 d with continuous passages and labeled as D9 (xylose before memory). It was then transferred to SX medium for 6 d with continuous passages and labeled as D9X6 (xylose after memory). It was finally transferred to SG medium for 9 d and labeled as D9X6D9. (xylose memory disappearance). The cells were cultured at 30℃ and 200 rpm. Cells at the middle of the logarithmic growth cycle were harvested. We used an integrated approach involving TMT labeling, HPLC fractionation,

Kac antibody affinity enrichment, and LC-MS/MS to quantify the dynamic changes in the whole acetylome. The quantitative ratio over 1.2 was considered up-regulation while a quantitative ratio below 0.83 was considered as down-regulation. To further understand the function and feature of the identified and quantified proteins, we performed gene annotation based on different categories such as gene ontology (GO), domain, pathway, and subcellular localization. Both the identified and quantifiable proteins were annotated. Comparison group-based clustering was performed for D9X6 vs. D9, D9X6D9 vs. D9, and D9X6D9 vs. D9X6 groups. Bioinformatics analyses such as GO annotation, domain annotation, subcellular localization, Kyoto Encyclopedia of Genes and Genomes pathway annotation, and functional cluster analysis were performed to annotate the quantifiable lysine-acetylated targets in response to drug treatment. Based on the results, further studies, including quantitative lysine acetylome analysis, were performed.

XCM-related acetylation site prediction

Before training the dataset, we preprocessed the assay data, considering that each state has only 3 samples, and if a test value is missing for one sample, several samples of that type will not have the value of that gene. Therefore, these genes were excluded from the analysis. Partial least square (PLS) is an efficient statistical classification technique suitable for analyzing high-dimensional data and genomic and proteomic data, especially for the problems of classification and dimension reduction in bioinformatics and genomics[59]. PLS is a commonly used feature extraction algorithm. This algorithm is based on the idea of latent variables that model the relationship between the input variable Xn×m (n: loci, m: samples) and the response variable Y1×m (in the case of D9 vs. D9X6, Y is a column vector like [1, 1, 1, –1, –1, –1], wherein D9 corresponds to 1 and D9X6 corresponds to –1). Instead of identifying the hyperplanes of

minimum variance between the response and independent variables, it identifies a linear classification model by projecting the predicted variables and the observed variables to a new lower space. This is highly suitable for the analysis of high-dimension, low-sample size data in bioinformatics and synthetic biology. For more convincing results, we explored the VIP to calculate the importance of each site to the response variable, which is the basis for selecting the signature sites[60].

$$VIP=\sqrt{p\times(\boldsymbol{q}/\mathrm{sum}(\boldsymbol{s}))} \quad \text{(S1)}$$

where p is the number of genes in the training dataset , and

$$\boldsymbol{s}=\mathrm{diag}(\boldsymbol{T}'\times\boldsymbol{T}\times\boldsymbol{Q}\times\boldsymbol{Q}') \quad \text{(S2)}$$

$$\boldsymbol{q}=\boldsymbol{s}'\times\boldsymbol{w} \quad \text{(S3)}$$

where the parameters $\boldsymbol{T}$, $\boldsymbol{Q}$, and $\boldsymbol{w}$ are calculated using PLS; $\boldsymbol{w}$ is the unitized form of $\boldsymbol{W}$.

Gene site-directed mutagenesis and deletions

Site-directed mutagenesis and knockout of genes were performed using clustered regularly interspaced short palindromic repeat-associated Cas9 nuclease. The protospacer adjacent motif (PAM) sequences of guide RNAs (gRNAs) were designed using E-CRISP Design (http://www.e-crisp.org/E-CRISP/designcrispr.html) [61]. The sequences of gRNAs used in this study are summarized in Additional file 1: Table S3. *HDA1* knockout of the yYST45 strain was performed. The *GFP* marker protein was first excised by Not1 digestion of the pYST06 plasmid, followed by the joining of PAM sequence together by polymerase chain reaction (PCR) using gRNA-HDA1–1-F and gRNA-HDA1–1-R primers, ligation to the pYST006 plasmid, and verification of the generated clone by sequencing. Two simultaneous PCR reactions were performed. One fragment was amplified using primers OE-P142–1-F/OE-P142–2-R, whereas the other was amplified using OE-P142–3-F and OE-P142–4-R primer pairs. These two fragments were then used as templates

and overlapped in the second PCR reaction to obtain full-length homologous arms. The ligated products carrying the gRNA sequence and the knockout homologous arm were then transformed into the Top10 receptor cells to obtain the corresponding plasmids. These plasmids were sequenced to ensure that the appropriate strains were obtained. The appropriate homologous arm fragments were obtained by Bam I and Hind III digestion, and the purified plasmids and homologous arm fragments were then transformed simultaneously into *S. cerevisiae* yYST218 to obtain the corresponding strains.

Stagnation rate, forgetting value and memory value

S. cerevisiae cultured in microfluidics，ccording to the first and second delay period, the stagnation rate is obtained.

$$\textit{Forgetting value}=\frac{A}{B} \tag{S4}$$

A is cell stagnation growth time during the first SX culture in microfluidic, **B** is cell stagnation growth time during the second SX culture in microfluidic.

These strains lost xylose metabolism memory were transferred to SX and cultured for 58 hours. The xylose consumption obtained was compared to obtain the xylose forgetting value.

$$\textit{Forgetting value}=\frac{C}{D} \tag{S5}$$

C is *S. cerevisiae* were cultured in SX continuously for 6 days and transferred to SX for 58 hours to measure the consumption of xylose., **D** is *S. cerevisiae* were cultured in SX continuously for 6 days, then transferred to SG for 8 days, and finally transferred to SX for 58 hours to measure the xylose consumption.

Compare the xylose consumption rate of strains after the xylose memory 24 h with strains before the xylose memory is generated.

$$Memory\ \ value=\frac{E}{F} \quad \text{(S6)}$$

E is *S. cerevisiae* were continuously incubated in SG for 8 d, then transferred to SX for 24 h. and finally transferred to SX for 24 hours to measure the xylose consumption., **F** is *S. cerevisiae* were cultured in SG continuously for 8 days and transferred to SX for 24 hours to measure the consumption of xylose.

Abbreviations

S. cerevisiae: *Saccharomyces cerevisiae*

XCM: Xylose Consumption Memory

PLS: Partial Least Squares

E. coli: *Escherichia coli*

SG: synthetic complete-glucose medium

SC-Ura: SC medium without uracil

SX: synthetic complete-xylose medium

TMT: Tandem Mass Tags

LC-MS/MS: Liquid Chromatography-tandem Mass Spectrometry

PAM: Protospacer Adjacent Motif

gRNAs: guide RNAs

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeat

Acknowledgements

Not applicable.

Authors' contributions

YST conceived and designed the study, performed experiments, analyzed data, and drafted the

manuscript. YYW, and QEH performed experiments. BZL, ZHL, ZZ and KS designed and supervised the research, and revised the manuscript. YJY supervised the project. All authors read and approved the final manuscript.

Funding

This work was funded by Tianjin Fund for Distinguished Young Scholars (19JCJQJC63300) and the National Natural Science Foundation of China (21576198).

Availability of data and materials

The datasets generated during this study are included in this published article and its Additional files 1, 2 and 3.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure captions

Figure 1 The construction of xylose-utilizing *S. cerevisiae* and their xylose consumption ability under different culture strategies in shake flask fermentation. A. Xylose metabolic pathway constructed in *S. cerevisiae*. B. The xylose consumption ability under different culture strategies, by which *S. cerevisiae* chassis cells were first cultured in synthetic complete-glucose medium (SG) or synthetic complete-xylose medium (SX) and then switched to SX. D represent *S. cerevisiae* was first cultured in SG and then switched to SX. X represent *S. cerevisiae* was first cultured in SX and then switched to SX again.

Figure 2 Microfluidic technology validated the 'xylose consumption memory (XCM)' behaviors

of *S. cerevisiae* in changing culture environments. A. Schematic representation of the microfluidic device enabled free switching of culture medium and the micrograph of cells grown inside the growth chambers. B. Schematic representation of the time course experiments to monitor the behavior changes of *S. cerevisiae* during carbon-source shifts. C. Microfluidic technology to detect the germination time of *S. cerevisiae* strains under different culture modes. D. Stagnation rates of *S. cerevisiae* strains detected by the microfluidic technology.

Figure3 The identification of protein acetylation in *S. cerevisiae* under different culture modes. A. The culture mode design and the cell sample acquisition flow chart. D9 indicates that the cells was cultured in synthetic complete-glucose medium (SG) for 9 d in continuous passages; D9X6 indicates that the cells was cultured in SG for 9 d in continuous passages and then the cells was transferred to synthetic complete-xylose medium (SX) for 6 d in continuous passages; D9X6D9 indicates that the cells was cultured in SG for 9 d, then the cells was transferred to SX for 6 d, and again the cells was transferred to SG for 9 d. B. Comparative plot of lysine acetylation site changes in the yYST218 strain under different culture modes. C. Least-squares method employed for analyzing the protein acetylation and variable importance index (VIP) plot according to the importance of the acetylation site.

Figure 4 The loss of lysine acetylation site function regulated the 'xylose consumption memory (XCM)' behaviors of *S. cerevisiae*. a. Culture mode design induced the forgetting of "XCM". The strains were continuously incubated in synthetic complete-xylose medium (SX) to induce the "XCM", and the strains were transferred to synthetic complete-glucose medium (SG) for 0, 8, 12, 16 and 20 d, respectively, and finally transferred to SX. B, C, D, E and F indicate the xylose consumption ability of the strains after the acetylation capacity loss at the acetylation sites of

yYST218 strain, H4K5R (yYST210) and H4K8R (yYST211). G. The strains were incubated in SX continuously to induce "XCM", and the strains were then transferred to SG for 0 and 8 d, respectively, and finally incubated in SX for 58h. A smaller forgetting value represents slower forgetting and thus enhanced memory. *P<0.05

Figure 5 The deletion of a single acetylation modifying enzyme regulated the 'xylose consumption memory (XCM)' behaviors of *S. cerevisiae*. A. The culture mode induced the "XCM" of *S. cerevisiae*. Xylose consumption was detected by transferring the cells to synthetic complete-xylose medium (SX) after incubation in synthetic complete-glucose medium (SG). Then xylose consumption was detected by transferring to SX after incubation in SX for 24h. B, C, D, E, F, G, H and I are the knockdown of deacetylase and acetylase regulated the xylose consumption. D is the xylose consumption of the strains after incubation in SG for 24 h and SX. X is the xylose consumption of the strains after incubation in SX for 24 h and SX. J is the memory value of the acetylation-related enzyme knockdown assay, the larger the memory value the faster the memory, thus enhancing the memory. *P<0.05, ***P<0.001.

Supplementary information

Additional files

Additional file 1:

Table S1. Yeast strains and plasmids used in this study

Table S2 Primers used in this work

Table S3 gRNAs used in this work

Additional file 2: Differentially_modified_statistics

Additional file 3: VIP

Figures

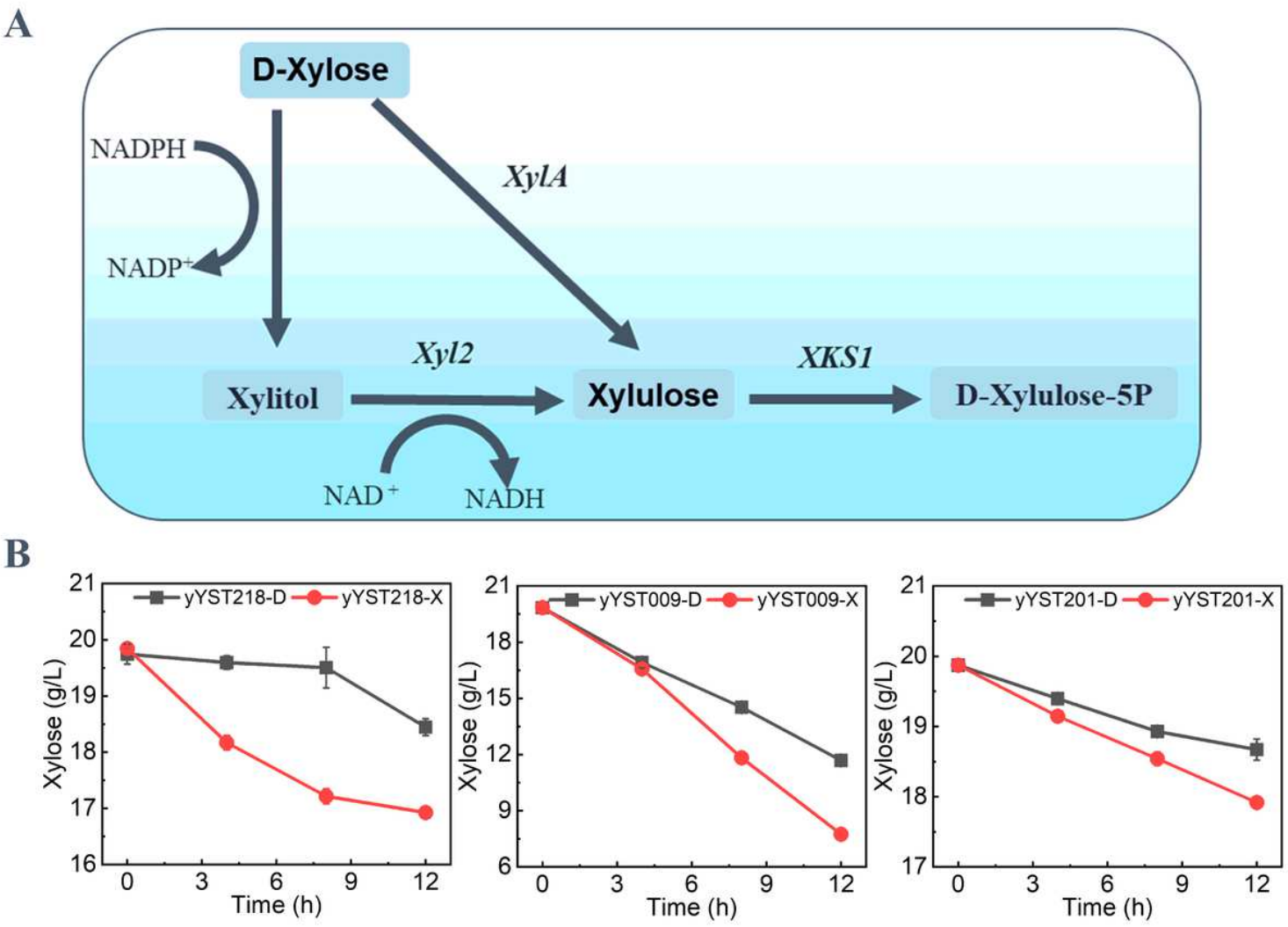

Figure 1

The construction of xylose-utilizing S. cerevisiae and their xylose consumption ability under different culture strategies in shake flask fermentation. A. Xylose metabolic pathway constructed in S. cerevisiae. B. The xylose consumption ability under different culture strategies, by which S. cerevisiae chassis cells were first cultured in synthetic complete-glucose medium (SG) or synthetic complete-xylose medium (SX) and then switched to SX. D represent S. cerevisiae was first cultured in SG and then switched to SX. X represent S. cerevisiae was first cultured in SX and then switched to SX again.

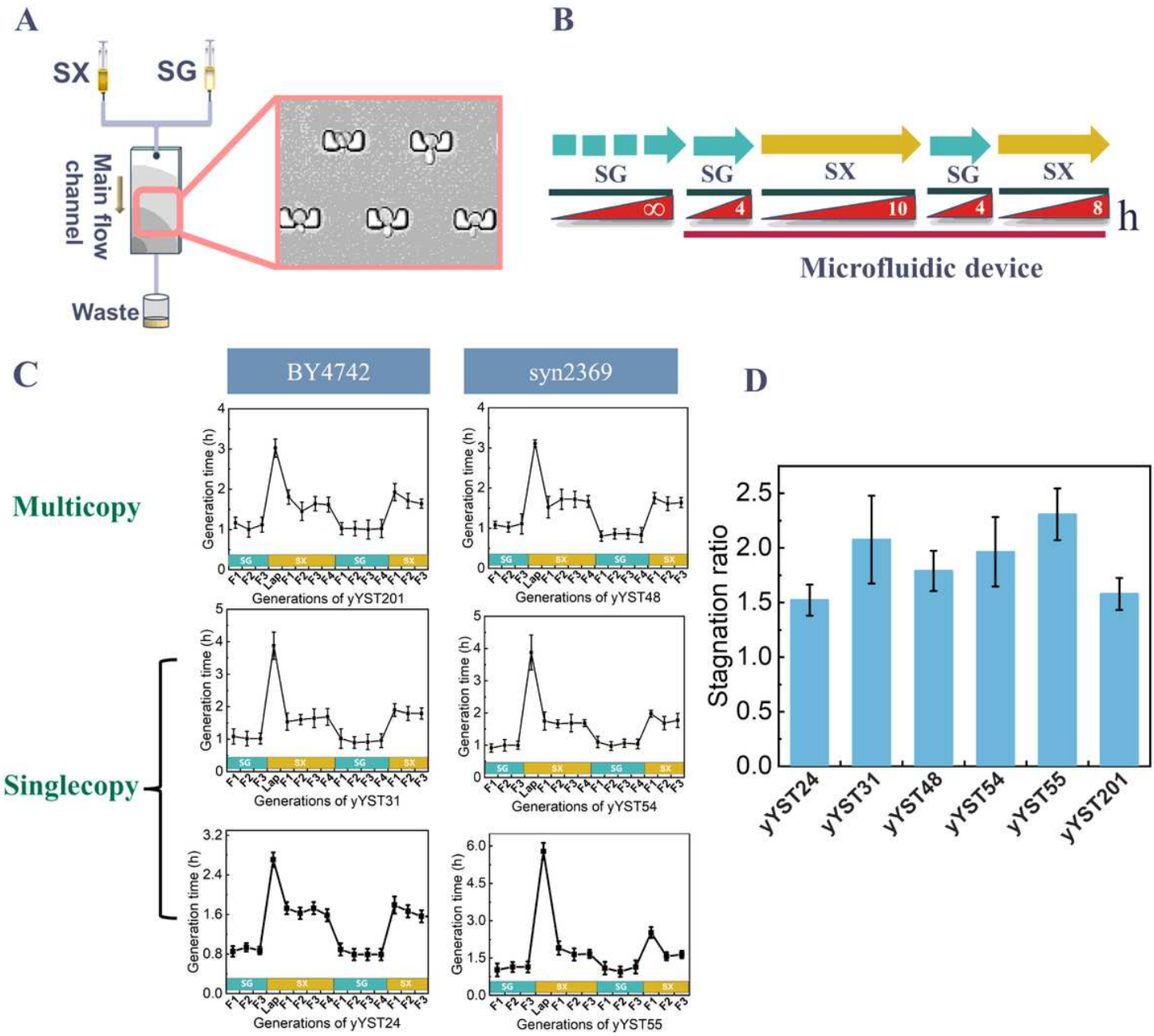

Figure 2

Microfluidic technology validated the 'xylose consumption memory (XCM)' behaviors of S. cerevisiae in changing culture environments. A. Schematic representation of the microfluidic device enabled free switching of culture medium and the micrograph of cells grown inside the growth chambers. B. Schematic representation of the time course experiments to monitor the behavior changes of S. cerevisiae during carbon-source shifts. C. Microfluidic technology to detect the germination time of S. cerevisiae strains under different culture modes. D. Stagnation rates of S. cerevisiae strains detected by the microfluidic technology.

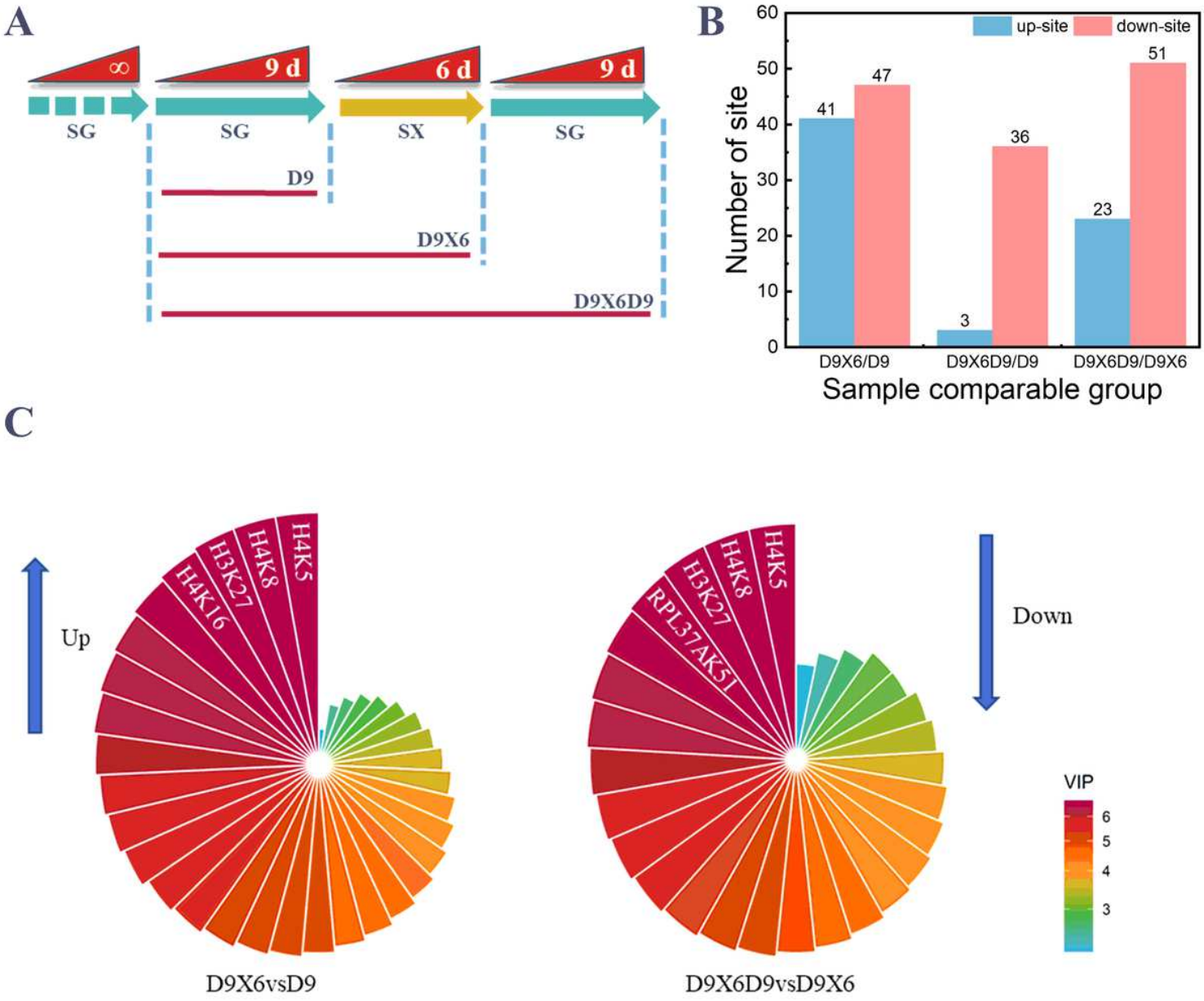

Figure 3

The identification of protein acetylation in S. cerevisiae under different culture modes. A. The culture mode design and the cell sample acquisition flow chart. D9 indicates that the cells was cultured in synthetic complete-glucose medium (SG) for 9 d in continuous passages; D9X6 indicates that the cells was cultured in SG for 9 d in continuous passages and then the cells was transferred to synthetic complete-xylose medium (SX) for 6 d in continuous passages; D9X6D9 indicates that the cells was cultured in SG for 9 d, then the cells was transferred to SX for 6 d, and again the cells was transferred to SG for 9 d. B. Comparative plot of lysine acetylation site changes in the yYST218 strain under different culture modes. C. Least-squares method employed for analyzing the protein acetylation and variable importance index (VIP) plot according to the importance of the acetylation site.

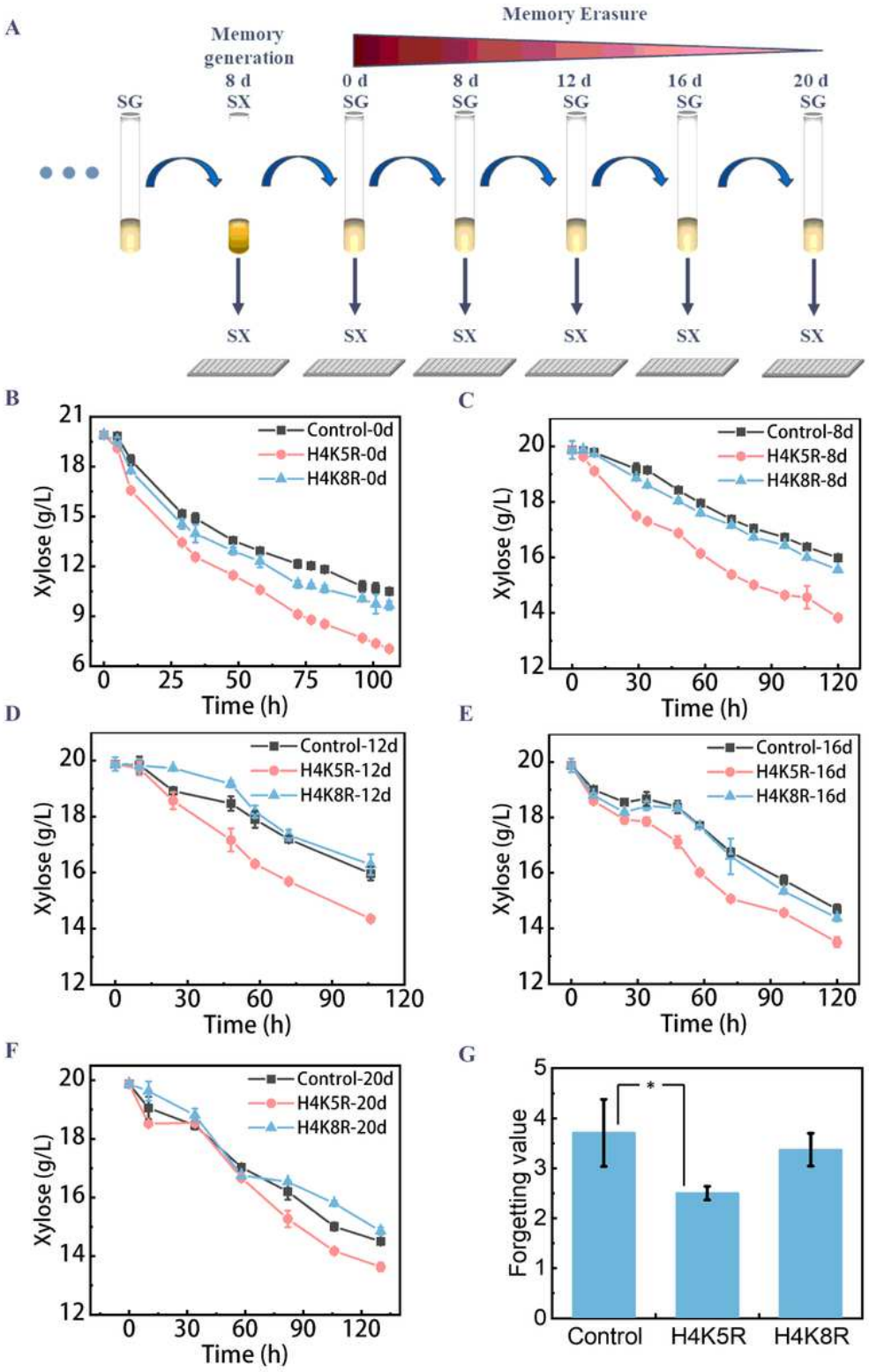

Figure 4

The loss of lysine acetylation site function regulated the ‘xylose consumption memory (XCM)’ behaviors of S. cerevisiae. a. Culture mode design induced the forgetting of “XCM”. The strains were continuously incubated in synthetic complete-xylose medium (SX) to induce the “XCM”, and the strains were transferred to synthetic complete-glucose medium (SG) for 0, 8, 12, 16 and 20 d, respectively, and finally transferred to SX. B, C, D, E and F indicate the xylose consumption ability of the strains after the

acetylation capacity loss at the acetylation sites of yYST218 strain, H4K5R (yYST210) and H4K8R (yYST211). G. The strains were incubated in SX continuously to induce “XCM”, and the strains were then transferred to SG for 0 and 8 d, respectively, and finally incubated in SX for 58h. A smaller forgetting value represents slower forgetting and thus enhanced memory. *P<0.05

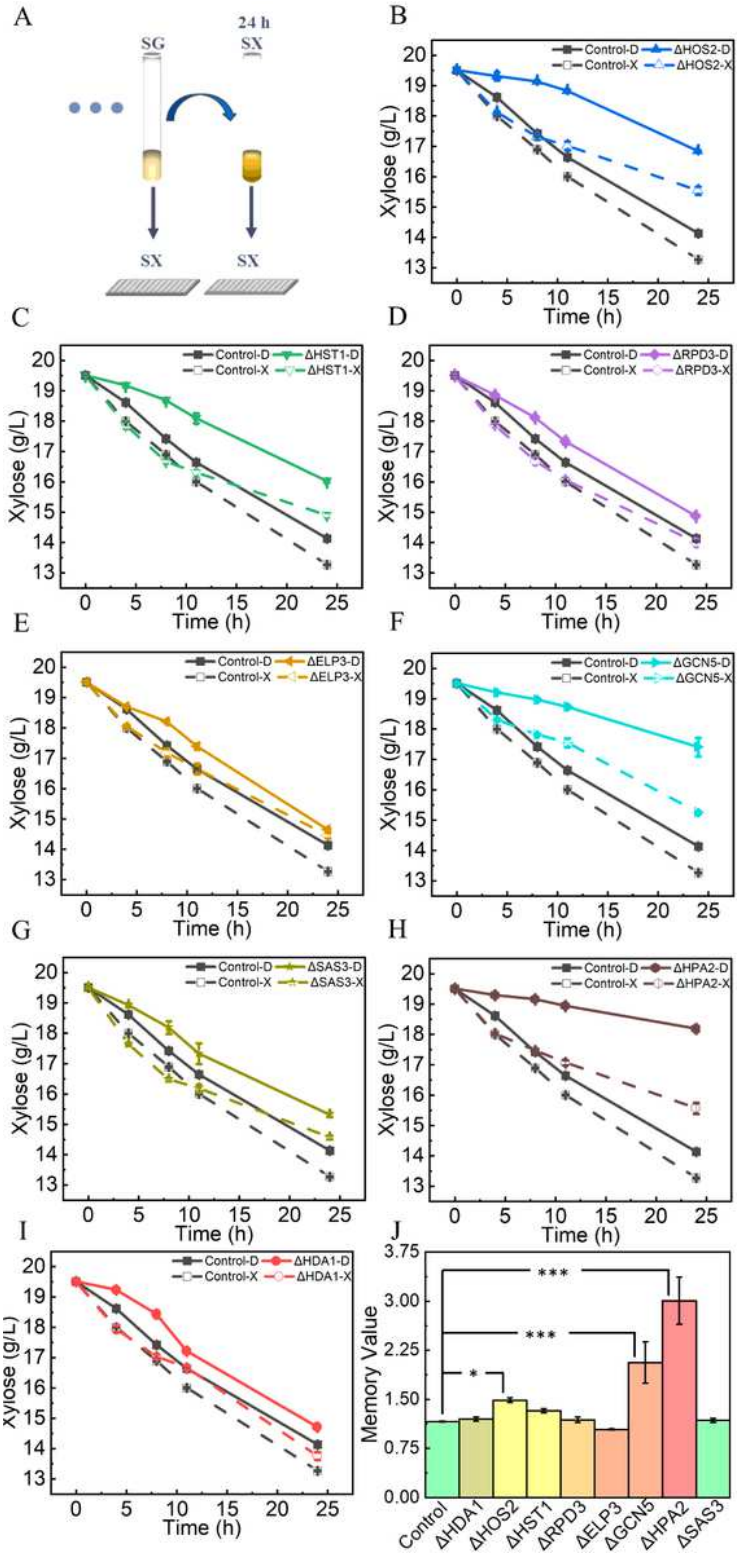

Figure 5

The deletion of a single acetylation modifying enzyme regulated the ‘xylose consumption memory (XCM)’ behaviors of S. cerevisiae. A. The culture mode induced the “XCM” of S. cerevisiae. Xylose consumption was detected by transferring the cells to synthetic complete-xylose medium (SX) after incubation in synthetic complete-glucose medium (SG). Then xylose consumption was detected by transferring to SX after incubation in SX for 24h. B, C, D, E, F, G, H and I are the knockdown of deacetylase and acetylase regulated the xylose consumption. D is the xylose consumption of the strains after incubation in SG for 24 h and SX. X is the xylose consumption of the strains after incubation in SX for 24 h and SX. J is the memory value of the acetylation-related enzyme knockdown assay, the larger the memory value the faster the memory, thus enhancing the memory. *P<0.05, ***P<0.001.

Supplementary Files

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- Additionalfile1.docx
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