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CHAPTER 6

Pichia anomala 29X: A Resistant Strain for Lignocellulosic Biomass Hydrolysate Fermentation

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Abstract

To efficiently use lignocellulosic biomass hydrolysates as fermentation media for bioethanol production, besides being capable of producing significant amount of ethanol, the fermenting host should also meet the following two requirements: (1) resistant to the inhibitory compounds formed during biomass pretreatment process, (2) capable of utilizing C5 sugars, such as xylose, as carbon source. In our lab, a screening was conducted on microorganisms collected from environmental sources for their tolerance to hydrolysate inhibitors. A unique resistant strain was selected and identified as *Pichia anomala* (*Wickerhamomyces anomalus*), deposited as CBS 132101. The strain is able to produce ethanol in various biomass hydrolysates, both with and without oxygen. Besides, the strain could assimilate xylose and use nitrate as N-source. These physiological characteristics make *Pichia anomala* an interesting strain for bioethanol production from lignocellulosic biomass hydrolysates.

Introduction

The future feedstock for bioethanol production is lignocellulosic biomass, which is abundant, renewable and inexpensive [1]. The typical procedure for bioethanol production from biomass consists biomass pretreatment and hydrolysis, hydrolysate fermentation, and ethanol separation [2]. In the pretreatment process, harsh conditions are used to break down the structure of biomass and expose cellulose and hemicellulose for hydrolysis. The harsh conditions also cause the formation of compounds that inhibit the hydrolysis as well as fermentation process [3-5]. After the pretreatment, the biomass is hydrolyzed into a mixture containing high concentrations of glucose and xylose, low amounts of galactose, arabinose and mannose, and inhibitory compounds [6]. This mixture, so-called hydrolysate, is used as fermentation medium for ethanol production.

To transform sugars in hydrolysates to ethanol with high efficiency, selection of the appropriate microorganism(s) is of high importance. Based on the hydrolysate composition, it is suggested that the desired microorganism(s) is to meet the requirements of being able to utilize glucose as well as xylose as carbon source and being resistant to hydrolysate inhibitors. The current ethanol production host is baker's yeast *Saccharomyces cerevisiae*, which converts glucose to ethanol with high rate [7]. However, native *S.cerevisiae* is not able to use xylose, and although efforts have been made to engineer this host for xylose consumption [8,9], until now the efficiency remained unsatisfying in feedstock hydrolysates [10-12]. In contrast to *S.cerevisiae*, *Scheffersomyces stipitis* is able to use xylose as carbon source for ethanol production [13,14], but its growth rate is very low when hydrolysate inhibitors are present [15,16]. These limitations make it interesting to discover new microorganisms, which meet both requirements for fermenting biomass hydrolysates.

In this study, we attempted to isolate strains from diverse environmental sources, and to select the ones that show resistance to hydrolysate inhibitors. Subsequently, the identification of the selected strains was carried out and their fermentation performance was analyzed.

Materials and Methods

Strain isolation

The environmental sources for isolating microorganisms are listed in Table 1. Briefly, these sources were suspended in physiological salt solution individually and incubated at 25°C, 150 rpm for 2 hours. The incubated suspensions were filtrated, 40 ml filtrates were collected and centrifuged. After centrifugation, the pellets were re-suspended in 4 ml fresh physiological salt solution. These solutions were then plated on plates with tryptone soya agar (Oxoid CM0131), Schaedler agar (Oxoid CM0437), and Oxytetracycline glucose yeast extract agar (Oxoid CM0545). The tryptone soya agar and oxytetracycline glucose yeast extract agar plates were incubated at 30°C aerobically, while schaedler agar and oxytetracycline glucose yeast extract agar plates were used to cultivate microaerophilic microorganisms. In total 184 colonies were selected and inoculated in 96-well microtiter plates with 0.5 ml tryptone soya broth as medium. The colonies that grew under aerobic conditions were incubated aerobically, and the microaerophilic colonies were incubated under microaerobic conditions.

The resistance of the isolated microorganisms to hydrolysate inhibitors was tested using 96-well microtiter plates. Mineral medium (MM) [17] was supplied with a mixture of 10 mM furfural, 10 mM HMF, 10 mM acetic acid, and 10% wheat straw hydrolysate prepared with a concentrated acid method [18]. The pH of the culture medium was adjusted to 5.0 before inoculation. The tests were conducted in duplicate under both aerobic and microaerobic conditions dependent on the original selection condition. The plates were incubated at 30°C, 100 rpm for 5 days, and the growth was checked either visually or by measuring optical density (OD) of each well using Infinite® F500 (TECAN). The strains growing in this medium were analyzed for their identities by sequencing the D2 domain of large subunit (26S) rRNA genes (the D2-LSU procedure: BASECLEAR, Leiden, the Netherlands).

Strains, biomass hydrolysates and growth test

Pichia anomala (*Wickerhamomyces anomalus*) 29 (CBS134880), 32, 35 and *Pichia burtonii* (*Hyphopichia burtonii*) were the strains isolated in this study. *P. anomala* 29X (CBS132101), was selected as a xylose consuming derivative from *P. anomala* 29. *P. anomala* CBS 1984 and CBS 5759, and *Saccharomyces cerevisiae* CEN.PK 113-7D (CBS8340) were purchased from CBS (Utrecht, the Netherlands), and *P. anomala* J121 (CBS 100487) was kindly provided by Dr. Passoth (SLU, Sweden).

The biomass used were sugar cane bagasse (Bag) (Zilor, Brazil), corn stover (CS) (University of Cape Town, South Africa), wheat straw (WS) (Oostwaardshoeve, The Netherlands), and oak sawdust (Oak) (wood-flooring supplier ESCO, The Netherlands). Prior to pretreatment, biomass (except Oak) was ground to pieces of average length 3 mm and dried at 80 °C for at least 16 hours. To prepare 1 l hydrolysate, 300 g dried biomass was used. The pretreatment methods were dilute acid (DA) (2% H₂SO₄), and concentrated acid (CA) (72% H₂SO₄). The pretreated biomass was hydrolyzed enzymatically, using Accellerase 1500 (Genencor[®]) [18]. After hydrolysis, solid content was separated from the hydrolysate by filtration, and the filtrated hydrolysate was sterilized using filter sterilization and stored at 4 °C before use.

Growth tests were conducted in honeycomb plates, using Bioscreen C Analyzer (Labsystems OY). MM with 20 g/l glucose was used as reference medium, into which the selected inhibitors were added individually with the concentrations listed in Table 2. The detailed procedure of Bioscreen C test is described in Zha *et al.* [18]. All tests were carried out in triplicate.

Fermentation

Batch fermentations were carried out in 2 l New Brunswick fermentors, using 1 l of either MM with 60 g/l glucose and 30 g/l xylose or sterilized hydrolysate as substrate. The strain used for fermentation was *P. anomala* 29X (CBS 132101), and the inoculum was prepared in a 500 ml Erlenmeyer flask. The cells were harvested by centrifugation after incubating 40 hours in MM with 20 g/l xylose as carbon source, and inoculated into fermentors with density of 0.2 g cell dry weight per 1 l medium. All fermentations were carried out at 30 °C, and pH 5 by adding 1 M H₂SO₄ or 2 M KOH. The anaerobic condition was created by sparging 0.5 l/min N₂ continuously, and 20% air was mixed into the gas-in flow when oxygen was required. During the whole fermentation process, CO₂ concentration in the off-gas was monitored automatically and samples were taken at fixed time intervals. These samples were kept at 4 °C and used to measure OD, glucose and ethanol concentration with Arena[®] 20 Analyzer (Thermo Scientific), and xylose with YSI 2700 SELECT Biochemistry Analyzer.

Results and Discussion

Resistant strain isolation

With the purpose of discovering natural isolates that are resistant to the inhibitory compounds in biomass hydrolysates, diverse environmental sources were collected and explored to obtain bacterial, yeast and fungal colonies. In total, 184 colonies were obtained from 10 sources, as listed in Table 1.

Table 1 Colonies obtained from the 10 environmental sources collected in our lab.

| source type | bacteria | | yeast / fungi | |
|----------------------------------|----------|--------------|----------------------------|----------------|
| | aerobic | microaerobic | aerobic | microaerobic |
| fresh cow rumen fluid | 7 | 7 | 0 | 0 |
| fermented cow rumen fluid | 6 | 6 | 0 | 0 |
| garden compost | 6 | 7 | 5 | 5 |
| vegetable/fruit compost | 6 | 4 | 0 | 3 |
| mushroom compost | 13 | 10 | 3 (1) ^a | 1 (1) |
| grass silage | 8 | 8 | 6 (2^b) | 4 (2) |
| goat manure | 3 | 4 | 3 | 4 (1) |
| sewage | 7 | 6 | 4 (1) | 4 (1) |
| industrial anaerobic waste water | 6 | 4 | 1 | 2 (1) |
| industrial aerobic waste water | 8 | 8 | 2 | 3 (1) |
| total | 70 | 64 | 24 | 26 |

^a Strains in () grew with addition of 10 mM furfural, 10 mM HMF, 10 mM acetic acid and 10% wheat straw hydrolysate; ^b The four strains in **bold** showed the highest OD increase.

To select colonies that show resistance to biomass hydrolysate inhibitors, furfural, HMF and acetic acid were used as carbon source in MM for growth screening. These three compounds are the representatives of inhibitors in biomass hydrolysates [19-21]. In a final screen, 11 strains grew in a mixture of furfural, HMF, acetic acid and 10% wheat straw hydrolysate, among which four showed clear OD increase (Table 1). Interestingly, the corresponding colonies of these four strains were all isolated from grass silage. Of these four, based on partial 26S sequencing (2D-LSU), three isolates (#29,32,35) were identified as *Pichia anomala*, (*Wickerhamomyces anomalus*), one as *Pichia burtonii* (*Hyphopichia burtonii*; isolate #34). DNA sequence Accession numbers of the sequenced partial 26S rRNA genes of the four isolates are HF952836 (isolate #29), HF952837 (isolate #32), HF952838 (isolate #35), and HF952839 (isolate #34).

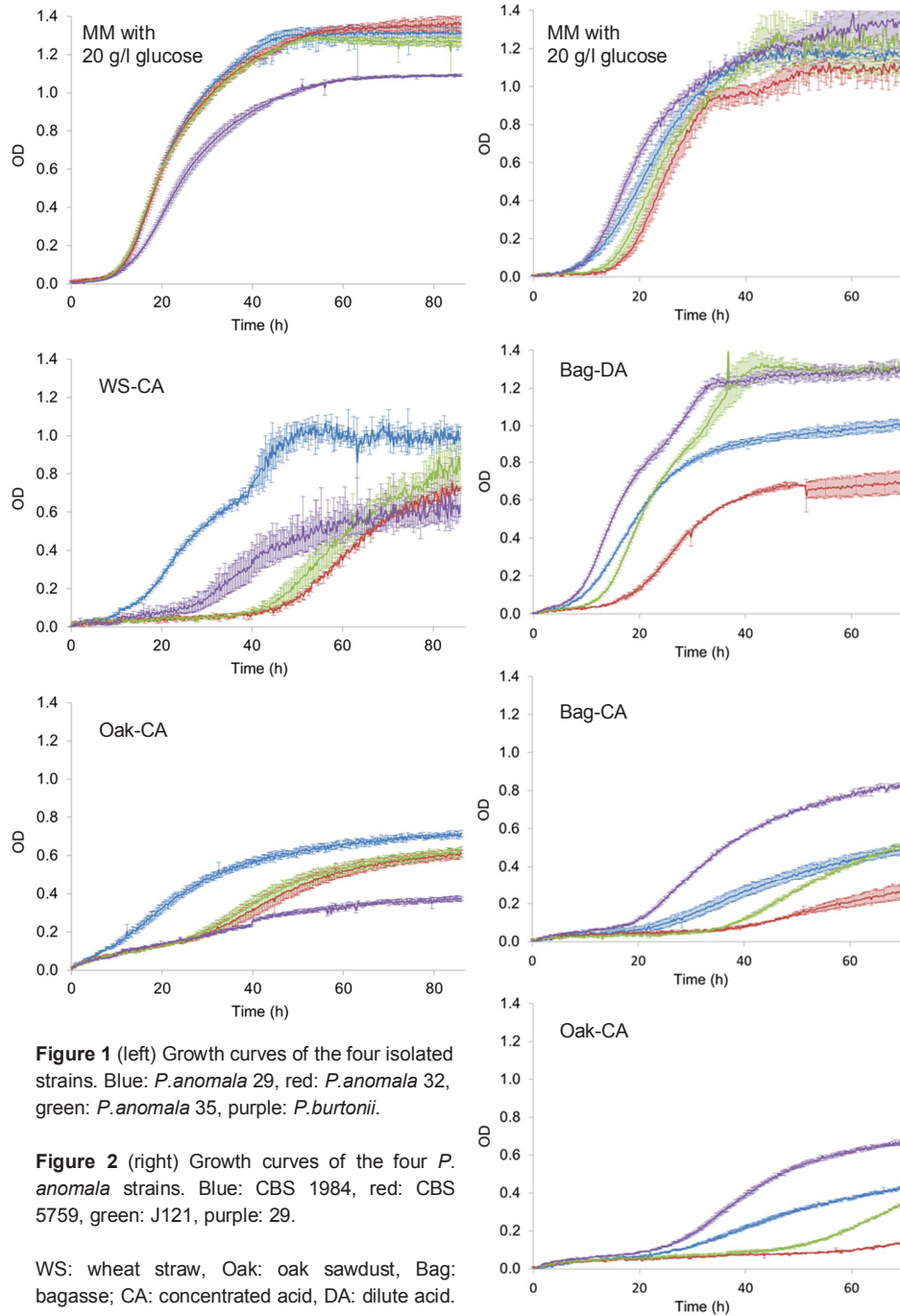
Resistant strain selection in hydrolysates

Interestingly, of the four most resistant strains isolated from grass silage, three were identified as *P. anomala* (*Wickerhamomyces anomalus*). This yeast was also found on insects and fruits [22,23], and is known for its ethanol and ethyl acetate production [24,25]. One of the most studied *P. anomala* strain was J121, its physiological characteristics and metabolite profiles were discussed in detail by Fredlund *et al.* [26,27]. Although there was a brief description on ethanol production by a *P. anomala* strain in alkaline hydrolyzed citrus peel [28], its ability to ferment diverse biomass hydrolysates and its resistance to biomass hydrolysate inhibitors was not studied.

In our study, the selected three *P. anomala* strains #29,32,35 and the *P. burtonii* strain were tested for their growth in hydrolysates prepared from WS and Oak. To demonstrate the resistance and difference of the four strains, hydrolysates pretreated with CA method were used as media, since it was shown that CA hydrolysates were most toxic to baker's yeast [18]. The growth tests were conducted in Bioscreen C Analyzer at 30°C in triplicate, and the results are shown in Figure 1. It can be seen that for all four strains, growth took place in both hydrolysates but with a lower rate than in MM with 20 g/l glucose. Strains were more sensitive in Oak-CA hydrolysate, which is more toxic compared to WS-CA ([18] and Figure 1).

Among the four *Pichia* strains, *P. anomala* 29 exhibited a unique tolerance to hydrolysate inhibitors, as its growth was considerably better. *P. burtonii* strain already showed a poorer growth performance in MM with 20 g/l glucose compared to the three *P. anomala* strains, which was even more the case in hydrolysates, indicating that *P. burtonii* is not a suitable potential fermenting yeast for biomass hydrolysates. The growth rate of *P. anomala* 32 and 35 was highly similar, but lower compared to strain 29 in both WS-CA and Oak-CA hydrolysates (Figure 1). Since *P. anomala* 29 performed better in hydrolysates compared to strain 32 and 35, it was suspected that *P. anomala* 29 has unique resistance to hydrolysate inhibitors. To verify this, three other *P. anomala* strains from culture collections, namely CBS 1984, CBS 5759 and J121, were tested for their growth in hydrolysates, together with *P. anomala* 29. CBS 5759 and its haploid derivative CBS 1984 were analyzed for their fermentation characteristics showing that their central carbon metabolism regulation was mainly influenced by oxygen availability [29]. *P. anomala* J121 was isolated from airtight stored grain and is used as a biocontrol yeast for preservation of moist grain [26].

Pichia anomala: a strain for fermenting biomass hydrolysates



The growth of these *P. anomala* strains was compared in MM with 20 g/l glucose and several different hydrolysates, as shown in Figure 2. In MM, their growth performance was comparable, though *P. anomala* 29 grew slightly faster. Different from in MM, in hydrolysates, clear growth variations were observed. In all tested hydrolysates, *P. anomala* 29 performed the best, had the shortest lag-phase, the fastest growth rate and reached the highest final OD. This indicates that *P. anomala* 29 is unique in its high resistance to the inhibitory compounds in biomass hydrolysates. It is also noticed that in hydrolysates with higher toxicity, i.e. ones prepared with CA method, the growth difference between *P. anomala* 29 and the other three strains was larger. In DA prepared hydrolysate, which is less toxic, the performance of J121 was similar to that of *P. anomala* 29. These results suggest that especially in hydrolysates with high toxicity, *P. anomala* 29 is a suitable fermenting yeast.

Xylose utilization

It is known from previous research that *Pichia anomala* is able to use xylose as carbon source [22]. Since xylose is the second most abundant sugar form in lignocellulosic biomass hydrolysates, it is important to study if *P. anomala* 29 is also able to utilize xylose.

For this purpose, *P. anomala* 29 was cultivated in shake flask with MM and 20 g/l xylose as sole carbon source. No growth was observed in the first 48 hours, after which a slight OD increase was shown. This indicates that *P. anomala* 29 is able to use xylose as carbon source, but with a much lower efficiency compared to glucose.

To improve the growth of *P. anomala* 29 on xylose, 2 ml of the xylose culture was transferred into another shake flask with fresh MM and 20 g/l xylose for growth. This procedure was repeated until growth was observed within 20 hours after inoculation. The *P. anomala* 29 derivative, referred to as 29X, with improved xylose consumption ability was deposited in CBS under number 132101, and used as the inoculum for all fermentations described below.

Fermentation

The small scale (400 μ l) growth tests demonstrated that *Pichia anomala* 29 is a potential resistant strain for biomass hydrolysate fermentation. Besides, the shake flask experiment showed that the strain is able to use xylose as carbon source, which is a highly favorable characteristic in fermenting biomass hydrolysates [30,31]. Another potential advantage of using *P. anomala* as fermentation host is that the contamination occurrence may be reduced due to its production of killer toxin [26,32]. Furthermore, the isolated *P. anomala* strain is able

to use NO_3 as N-source (data not shown), making it even more versatile in using substrates with low NH_4 or amino acid levels [26,33]. To initially understand the growth property of *P. anomala* 29X in a controlled environment, batch fermentations with working volume of 1 l were conducted in both MM with 60 g/l glucose and 30 g/l xylose, and hydrolysates, prepared with the CA method.

MM fermentations

Pichia anomala was described as having a Pasteur effect, which makes it oxygen sensitive instead of glucose sensitive, in terms of product formation [27,29]. Due to this, we initially conducted two parallel batch fermentations in MM, one anaerobically (100% N_2) and one, with 20% air (80% N_2). The carbon sources of these fermentations were 60 g/l glucose and 30 g/l xylose, which mimics the sugar composition of biomass hydrolysates [3]. The fermentations were monitored by measuring OD, glucose, xylose and ethanol concentrations of the samples taken with a fixed interval during the process, as shown in Figure 3 [26].

First of all, it can be seen that the strain is capable of growing both with and without oxygen supply, though the growth was much faster when oxygen was present (Figure 3a). The glucose consumption was completed within 20 hours when 20% air was mixed into the gas-in flow, while under strict anaerobic conditions, it took about 45 hours to consume all the glucose in MM (Figure 3b).

Secondly, a similar ethanol production pattern was observed in both fermentations, with and without oxygen supply. The amount of ethanol produced was also comparable. This challenges the claim that *P. anomala* as a species showing Pasteur effect [27,29], since no suppression on ethanol formation was observed when 20% air was added into the gas-in flow. However, as soon as glucose was depleted, ethanol was subsequently consumed when oxygen was present. It was suspected that besides producing biomass, ethanol was partially converted into ethyl acetate (EA), since small amount of EA was detected in the fermentor (~ 5 g/l). The production of EA by *P. anomala* was also reported by previous studies as a mechanism of forming anti-fungal compounds [34,35] or producing flavor component during wine making process [36,37]. EA was also produced as the main product through biotechnological process using *P. anomala* on a pilot scale, as EA is a widely used environmentally friendly solvent [38]. Therefore, besides ethanol, *P. anomala* can also be used potentially for EA production with biomass hydrolysate as substrate.

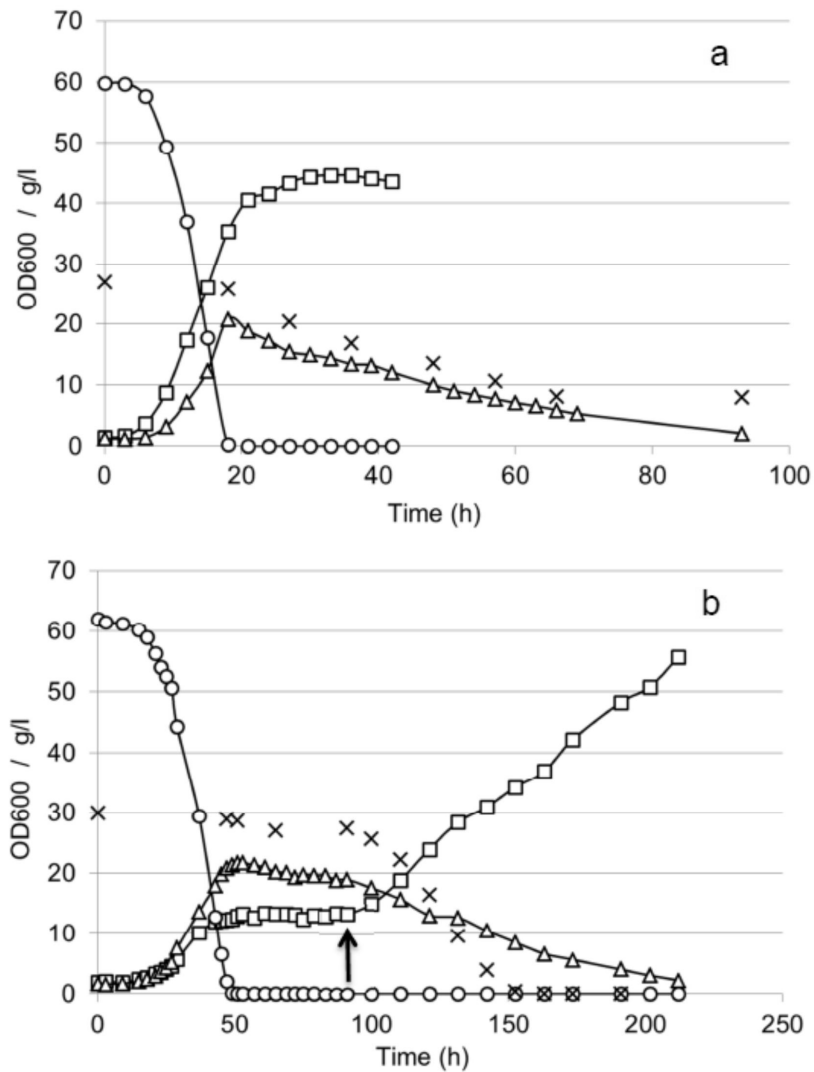


Figure 3 Fermentation results of *Pichia anomala* 29X (CBS132101) in MM (60 g/l glucose and 30 g/l xylose), square: OD, circle: glucose, cross: xylose, triangle: ethanol. (a) 20% air + 80% N₂, (b) 100% N₂ to 20% air + 80% N₂, the time-point when 20% air was added into the gas-in flow is indicated by an arrow.

Thirdly, the strain was capable of assimilating xylose after glucose was depleted (Figure 3a), which is a characteristic that was reported for some but not all *P. anomala* isolates earlier [39]. We confirmed the presence of xylose reductase, xylitol dehydrogenase and D-xylulokinase in *P. anomala* strain by detecting homologs of the corresponding genes of *P. stipitis* in its

genome. (xylose reductase: GenBank ADQ89193.1, xylitol dehydrogenase: GenBank AAD28251.1, D-xylulokinase: GenBank EAZ63302.2; *P. anomala* genome database: <http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=885923>). However, no xylose consumption was observed under anaerobic conditions, and only when air was mixed into the gas-in flow, a simultaneous xylose decrease and OD increase took place (Figure 3b). This indicates that the *P. anomala* strain is not xylose fermenting, probably due to the redox imbalance that caused by the overproduction of NADH in the xylose reduction step [40]. This feature is undesirable in the context of ethanol production from biomass hydrolysates.

Finally, as described above, the produced ethanol was consumed / converted after glucose was depleted in the fermentor, which took place simultaneously with the consumption of xylose, after air was added into the gas-in flow (Figure 3b). To prevent losing ethanol, the following strategies could be considered (1) keep the fermentation anaerobic, and engineer and/or adapt the strain to ferment xylose under anaerobic conditions; (2) stop the fermentation process as soon as glucose consumption is complete, by temperature drop for instance, then recover ethanol from the fermentation broth, and continue fermenting the remaining xylose by a pentose fermenting strain [41]; (3) *in-situ* ethanol recovery, that is to remove ethanol during the fermentation process continuously. It is highly likely that an evolutionary adaptation process is also needed to improve the ethanol productivity of the *P. anomala* strain [42].

Hydrolysate fermentations

From the fermentation results of *P. anomala* 29X in MM with 60 g/l glucose and 30 g/l xylose, it was clear that the strain produced ethanol using glucose both with and without oxygen, while only when oxygen was present, xylose and ethanol consumption took place (Figure 3). Based on these observations, a two-stage fermentation procedure was adopted to test the performance of *P. anomala* 29X in hydrolysates. The two-stage fermentation started with anaerobic conditions (100% N₂) to allow the production of ethanol with glucose present in the hydrolysates. As soon as glucose was depleted, indicated by a sudden CO₂ drop in the off-gas, 20% air was mixed into the gas-in to allow the utilization of xylose.

To test the resistance of *P. anomala* 29X to the inhibitory compounds in hydrolysates, CA pretreated hydrolysates were used as fermentation media. Since CA hydrolysates had higher inhibitory effects compared to the ones prepared with other methods, like mild alkaline and diluted acid [18], the two CA hydrolysates used here were made from WS and CS, as shown in Figure 4.

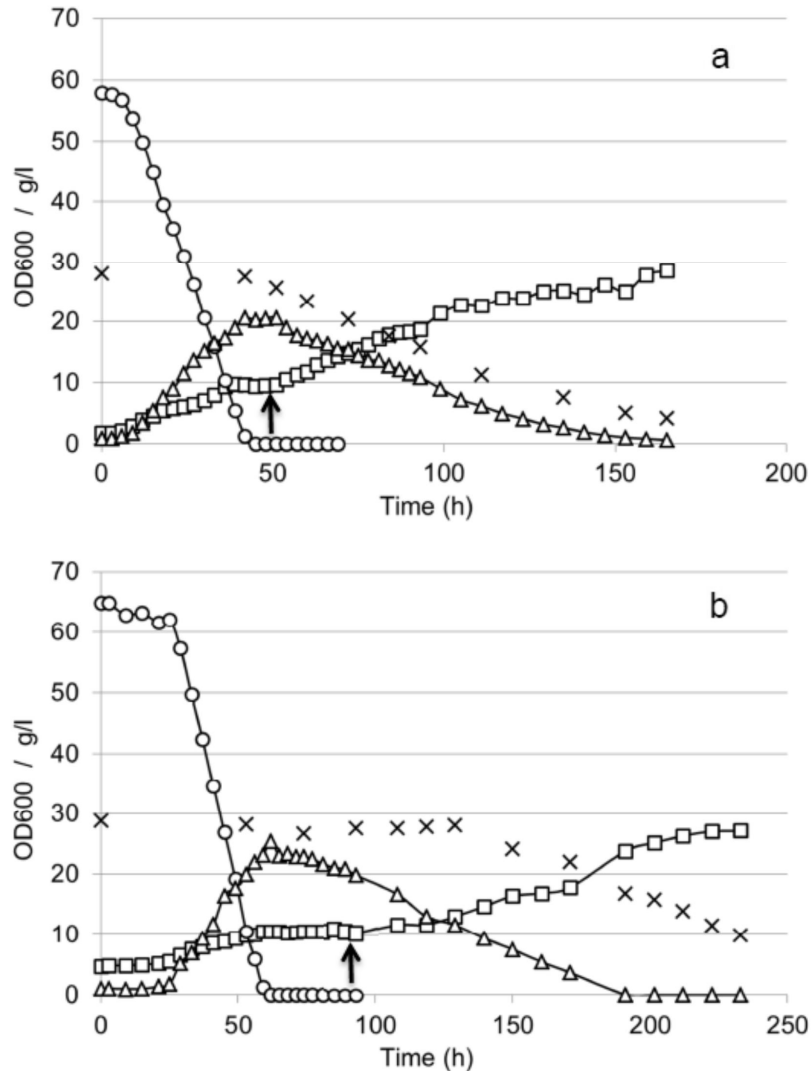


Figure 4 Fermentation results of *Pichia anomala* 29X in concentrated acid prepared hydrolysates, aeration conditions: 100% N₂ to 20% air + 80% N₂, the time-point when 20% air was added into the gas-in flow are indicated by arrows, square: OD, circle: glucose, cross: xylose, triangle: ethanol. (a) wheat straw hydrolysate (WS-CA), (b) corn stover hydrolysate (CS-CA).

It can be seen that very similar to its performance in MM (Figure 3b), *P. anomala* 29X strain rapidly consumed all the glucose in WS-CA hydrolysate under anaerobic conditions, no xylose or ethanol consumption was observed at this stage (Figure 4a). As soon as air was mixed into the gas-in flow, indicated by the black arrow in Figure 4a, growth took place again,

while ethanol as well as xylose started to decrease. The similar fermentation performance as in MM suggests that *P. anomala* 29X had considerably high resistance to the inhibitors in WS-CA hydrolysate, in terms of ethanol production from glucose.

As far as xylose utilization is concerned, the strain seems to be more sensitive to the inhibitors, since the xylose consumption was much slower compared to that in MM (Figure 4a and 3b). This was also observed with CS-CA hydrolysate, of which the xylose consumption was still incomplete after 233 hours (Figure 4b). The low inhibitor tolerance during the xylose fermentation process was reported in recombinant yeasts previously [11,43,44]. For instance, when less diluted hydrolysate was used as fermentation medium, xylose consumption rate decreased significantly, while glucose level was almost not influenced. Since this phenomenon was also observed with *P. anomala* 29X, a xylose utilizing isolate, it is suspected that the inhibitory mechanism may relate to the enzymes involved in the xylose consumption pathways.

Compared to WS-CA, CS-CA was a more toxic hydrolysate when tested with *S.cerevisiae* (see Chapter 5). In this study, the *P. anomala* 29X strain showed also less resistance to the CS-CA hydrolysate, indicated by a longer initial lag-phase and a slower glucose consumption rate (Figure 4b). Moreover, after air was mixed into the gas-in flow, no growth took place for a period of 27 hours, and only after 67 hours, xylose started to decrease (Figure 4b). This suggests that the resistance of *P. anomala* 29X is hydrolysate dependent, which was also shown in small scale growth experiments (Section Resistant strain selection in hydrolysates). Therefore, when hydrolysates with very high inhibitor concentration are used as fermentation substrates, dilution and/or detoxification still need to be applied to allow a reasonable performance of the yeast strain.

Resistance test

To further understand the resistance of *P. anomala* 29X strain to individual inhibitory compounds in biomass hydrolysates, an initial growth test was conducted using MM with 20 g/l glucose as reference medium, and *S.cerevisiae* CEN.PK 113-7D as reference strain. A series of potential inhibitory compounds were chosen, as shown in Table 2. They were either quantitatively detected in biomass hydrolysates [3], or were shown to be decreasing in hydrolysate fermentation with *S.cerevisiae* CEN.PK 113-7D (unpublished data). The concentrations used were relevant to that in actual hydrolysates [3] (Table 2).

Compared to *S.cerevisiae*, *P.anomala* 29X exhibited higher tolerance to furfural, benzoic acid, ferulic acid and salicylic acid (shaded with grey in Table 2), which are common inhibitors in biomass hydrolysates [5,45-47]; while for the other tested compounds, both strains showed similar resistance. This suggests again that *P.anomala* 29X is a potentially interesting strain for bio-product formation using lignocellulosic biomass hydrolysates as feedstock.

Table 2 Results of strain resistance test.

| In reference medium ^a | | <i>P. anomala</i> 29X | | | <i>S. cerevisiae</i> CEN.PK 113-7D | | |
|----------------------------------|---------------------|-----------------------|--------------------|------------------|---------------------------------------|-------|-----------|
| | | LP ^b | GR ^c | OD ^d | LP | GR | OD |
| | | 5.5 h | 0.067 | 1.2 | 6 h | 0.108 | 1.2 |
| Inhibitor | Concentration (g/l) | LP | GR | OD | LP | GR | OD |
| Acetic acid | 4.0 | 8 h | < 60% ^e | --- ^f | 9.5 h | < 60% | < 80% |
| Benzoic acid | 0.2 | 10 h | < 40% | < 60% | 9 h | < 20% | < 60% |
| Ferulic acid | 0.2 | --- | < 80% | --- | --- | < 40% | --- |
| Formic acid | 0.6 | 15 h | < 60% | < 80% | 10 h | < 60% | --- |
| Furfural | 0.6 | 8.5 h | --- | --- | 10.5 h | --- | --- |
| Furoic acid | 0.2 | --- | --- | --- | --- | < 80% | --- |
| Phenylacetic acid | 0.2 | --- | < 80% | --- | --- | < 80% | --- |
| Salicylic acid | 0.2 | --- | < 60% | < 80% | --- | < 40% | < 60% |
| Vanillin | 0.2 | --- | < 80% | --- | 7.5 h | --- | --- |
| 2,4-dihydroxycinnamic acid | 0.2 | | | | | | |
| 3,4-dihydroxycinnamic acid | 0.2 | | | | | | |
| 4-hydroxybenzaldehyde | 0.2 | | | | | | |
| 4-Hydroxycinnamic acid | 0.2 | | | | | | |
| 4-hydroxyphenylacetate | 0.2 | | | | | | No Effect |
| HMF | 0.2 | | | | | | |
| Levulinic acid | 1.5 | | | | | | |
| Vanillic acid | 0.2 | | | | | | |

^a MM with 20 g/l glucose; ^b LP: lag-phase: time needed to reach 2% of the maximum OD (h); ^c GR: growth rate: determined from the linear part of the OD curve (OD/h); ^d OD: final OD; ^e values in <% are relative growth rate and final OD compared to the corresponding host in reference medium; ^f '---': no effect compared to reference medium.

Conclusion

A strain, resistant to biomass hydrolysate inhibitors, was isolated from grass silage and identified as *Pichia anomala* (*Wickerhamomyces anomalus*). We have shown that the strain is able to produce ethanol in multiple biomass hydrolysates with different toxicity levels, is capable of utilizing xylose for growth when supplied with air, and can use nitrate as N-source. These characteristics makes *Pichia anomala* a potential ethanol production host using lignocellulosic biomass as feedstock. Further studies on the physiology of the yeast will help improve its fermentation performance in biomass hydrolysates.

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