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From lignocellulose to biofuels and chemicals – strain development and process optimization

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Abstract

Environmental concerns and limited fossil resources are driving the development of biorefinery technologies with the aim of gradually replacing petrochemical production of fuels and chemicals. However, the lignocellulose utilization process faces major techno-economic challenges. The recalcitrance of the lignocellulose requires the pretreatment of the raw material in order to facilitate efficient depolymerization of the structural carbohydrates cellulose and hemicellulose by enzymatic hydrolysis. The cost for the hemicellulolytic and cellulolytic enzymes thereby has a substantial impact on the overall process costs, necessitating intensification of the enzyme production process.

Trichoderma reesei is the principal enzyme producer but the complex morphology of the filamentous fungi presents a major challenge in the process development. We have developed a novel method for the analysis of the micromorphology of *T. reesei* in cultivations on lignocellulosic substrates, which, based on the solid loadings, precludes application of conventional light microscopy. Confocal laser scanning microscopy and computer-aided image analysis enabled the quantification of the dimensions of single cells and the degree of branching in cultivations on wheat straw and lactose. This study showed that the micromorphology of *T. reesei* was strongly dependent on the strain background, the employed substrate and the process conditions. The micromorphological changes were correlated with the efficiency of enzyme production. By providing a tool for the evaluation of the enzyme production by *T. reesei* on lignocellulosic substrate, this study has relevance for characterization and optimization of the critical step in the overall lignocellulose saccharification process.

In the second part of the biorefinery process the soluble sugars are converted to biofuels, e.g. bioethanol, or platform chemicals, e.g. lactic acid. The lignocellulosic hydrolyzates, however, present difficult substrates for the most commonly applied microorganism of the fermentation industries *Saccharomyces cerevisiae*. Secondary decomposition processes during pretreatment

of the raw material lead to the formation of toxic compounds such as furans, phenolic compounds and organic acids. The hydrolyzates further contain, besides the cellulose-derived sugar glucose, significant amounts of xylose, which is released from the hemicellulosic xylan. For efficient conversion of the sugars in the hydrolyzates to the desired product, strain development has to target at high inhibitor tolerance as well as efficient co-utilization of glucose and xylose. The herein presented *S. cerevisiae* strain IBB10B05 was enabled to xylose fermentation by the introduction of a NADH-dependent oxidoreductive xylose assimilation pathway within a CEN.PK113-7D background. Strain IBB10B05 was further evolved to accelerate xylose conversion rates and to promote anaerobic growth on xylose. The evolved strain IBB10B05 showed excellent xylose-to-ethanol conversion capabilities in fermentations of wheat straw hydrolyzates at high solid loadings. Compared to its parent strain BP10001, the specific uptake rate of strain IBB10B05 was up to 17.5-fold improved at complete perpetuation of the fermentation capabilities on glucose. Strain IBB10B05 therefore proved to be sturdy candidate for intensification of lignocellulose-to-bioethanol processes.

Based on the high robustness and the efficient xylose conversion, strain IBB10B05 was a promising host for recombinant L-lactic acid (LA) production. Selecting the *Plasmodium falciparum* L-lactate dehydrogenase (*pf*LDH) for its high kinetic efficiency, strain IBB14LA1_5 was derived from IBB10B05 by placing the *pfldh* gene at the *pdc1* locus under control of the *pdc1* promotor. By removing the *pdc5* gene, the pyruvate-to-ethanol pathway was disrupted. Strain IBB14LA1_5 was capable of continued LA formation under anaerobic conditions, provided that the pH was stabilized with added $CaCO_3$. The yields on both sugars were thereby advancing towards homolactate fermentation. With the aim of accelerating the xylose-to-LA conversion, cultivations by strain IBB14LA1_5 were further conducted under microaerophilic conditions, which increased the LA productivities significantly.

In recent years much research effort has been spent to optimize single unit operations and parameters in the biorefinery process. However, single parameters and process configurations

are heavily interrelated and can affect the overall process efficiency in a multitude of ways. Studies on process integration are therefore essential to render large scale biorefinery plants commercially viable. Despite this, comprehensive studies on process integration at an early stage of strain and process development, i.e. at laboratory scale, are lacking. We conducted an integrative analysis of a separate hydrolysis and co-fermentation process for the production of bioethanol on wheat straw. The process relied on the cultivation of a *T. reesei* mutant strain on the pretreated feedstock for the production of the hemicellulolytic and cellulolytic enzymes, and the efficient mixed glucose-xylose fermentation by *S. cerevisiae* strain IBB10B05. Process configurations were varied and evaluated with respect to the overall process ethanol yield ($Y_{\text{Ethanol-process}}$). The study generated a fundamental understanding of the process and allowed the identification of key process parameters with pronounced impact on $Y_{\text{Ethanol-process}}$. The highest $Y_{\text{Ethanol-process}}$ obtained was 71.2 g ethanol per kg raw material, which is comparable to data available from pilot scale plants. This study proved that benchtop-process analysis can be useful to for process optimization.

The herein presented strategies for strain and process development have a high relevance for the optimization of key steps in the biorefinery processes. The strategies advance the potential for lignocellulose as carbon source for the sustainable production of biofuels and chemicals.

Zusammenfassung

Das zunehmende Umweltbewusstsein und die Verknappung fossiler Ressourcen fördern die Entwicklung von Bioraffineriekonzepten mit dem Ziel, die petrochemische Herstellung von Kraftstoffen und Plattformchemikalien sukzessive durch nachhaltigere und umweltfreundlichere Alternativen zu ersetzen. Die Realisierung großtechnischer Prozesse zur Verwertung von Lignozellulose steht dabei weiterhin vor großen prozesstechnischen und ökonomischen Herausforderungen. Eine Problemstellung ist die hohe Resistenz von Lignozellulose gegenüber chemischer oder enzymatischer Hydrolyse. Um eine effiziente Depolymerisierung der strukturgebenden Naturpolymere Zellulose und Hemizellulose zu gewährleisten, muss das Rohmaterial vorbehandelt werden. Die Zellulasen und Hemizellulasen stellen einen signifikanten Anteil der Prozesskosten dar, weshalb eine Prozessintensivierung der Enzymproduktion notwendig ist. Zur Herstellung dieser Enzyme wird meist der filamentöse Pilz *Trichoderma reesei* eingesetzt. Die komplexe Morphologie dieses Pilzes stellt jedoch eine Herausforderung für die Prozessentwicklung dar. In dieser Studie wird eine neue Methode zur Untersuchung der Mikromorphologie von *T. reesei* während der Kultivierung auf lignozellulose-haltigen Substraten präsentiert. Konfokale Laser-Scanning-Mikroskopie und die Bildanalyse mit einem am Institut entwickelten Programm ermöglichten die quantitative Analyse der Geometrie einzelner Zellen in der Hyphe und des Verästelungsgrads während der Kultivierung von *T. reesei* auf Weizenstroh oder Laktose. Die Mikromorphologie änderte sich signifikant in Abhängigkeit von der verwendeten *T. reesei*-Mutante, dem Substrat und der Prozessführung. Weiterhin konnte die Änderungen der Mikromorphologie mit der Effizienz der Enzymproduktion korreliert werden. Mit dieser neuen Analysemethode ist es möglich, die Mikromorphologie von *T. reesei* während der Kultivierung auf Lignozellulose zu charakterisieren. Diese Studie ist daher von Relevanz für die

Optimierung der Enzymproduktion, die einen kritischen Prozessschritt in der Verzuckerung von Lignozellulose darstellt.

Im zweiten Teil des Bioraffinerieprozesses werden die löslichen Zuckermonomere zu Biotreibstoffen, z.B. Bioethanol, oder Plattformchemikalien, z.B. Milchsäure, umgewandelt. Die verzuckerten Lignozellulose-Hydrolysate stellen dabei besondere Anforderungen an den am meisten verwendeten Mikroorganismus *Saccharomyces cerevisiae*. Aufgrund von Abbauprozessen während der Vorbehandlung des Rohstoffes werden toxische Stoffe wie Furane, Phenole und organische Säuren gebildet. Die Hydrolysate enthalten neben der Glukose, die durch die Verzuckerung von Zellulose entsteht, auch hohe Mengen an Xylose, welches durch die Hydrolyse des Hemizellulose-Polymers Xylan entsteht. Um eine effiziente Umsetzung des Hydrolysates zu dem gewünschten Produkt zu gewährleisten muss also ein robuster Stamm mit der Fähigkeit zur Co-Fermentation von Glukose und Xylose entwickelt werden. Der hier präsentierte *S. cerevisiae*-Stamm IBB10B05 basiert auf dem CEN.PK113-7D-Wildtypstamm, dem der NADH-abhängige oxidoreduktive Xylose-Abbauweg eingefügt wurde. Mit gerichteter Evolution wurde der Stamm weiter modifiziert, um eine schnellerer Xylose-Konversion und anaerobes Wachstum auf Xylose zu ermöglichen. Der evolvierte Stamm zeigte dabei eine ausgezeichnete Xylose-Aufnahmerate und Ethanol-Produktion auf Weizenstroh-Hydrolysaten mit hohem Festoffanteil. Im Vergleich zu dem Ausgangsstamm BP10001 konnte die spezifische Xylose-Aufnahmerate um das 17,5-fache gesteigert werden. Die Fermentationskapazität auf Glukose blieb dabei unverändert. IBB10B05 stellt einen robusten Stamm für die Intensivierung der Bioethanol-Produktion dar.

Die hohe Stabilität macht IBB10B05 zu einem vielversprechenden Ausgangsstamm für die rekombinante Herstellung von L-Laktat. Dabei wurde – aufgrund der hohen kinetischen Effizienz – die L-Laktat Dehydrogenase von *Plasmodium falciparum* (*pf*LDH) verwendet. Die *pf*LDH wurde an den *pdc1*-Lokus unter der Kontrolle des nativen *pdc1*-

Promotors in den Stamm IBB10B05 kloniert. Um die Ethanol-Produktion zu eliminieren wurde das für die *pdc5* kodierende Gen deletiert. Der entstehende Stamm IBB14LA1_5 zeigte kontinuierliche anaerobe L-Laktat Produktion in gepufferten Glukose- und Xylose-Fermentationen. Der L-Laktat Ertrag auf beiden Zuckern war dabei fast so hoch wie der Homofermentativen Milchsäuregärung. Mit dem Ziel, die Fermentationsraten auf Xylose zu steigern, wurden die Fermentationen unter mikroaerophilen Bedingungen ausgeführt. Dies führte zu einer signifikanten Beschleunigung der L-Laktat Produktivität von IBB14LA1_5.

In den letzten Jahren hat sich die Forschung auf die Optimierung einzelner Prozessschritte oder Prozessparameter fokussiert. Da die einzelnen Prozesskonfigurationen aber stark miteinander vernetzt sind, wirkt sich die Veränderung eines Parameters in vielfacher Weise direkt oder indirekt auf die Effizienz des gesamten Prozesses aus. Um die großtechnische Umsetzung von Bioraffineriekonzepten zu realisieren, wären Studien zur Integration der einzelnen Schritte zu einem gesamten Prozess essentiell. Trotzdem sind umfassende Studien im frühen Stadium der Stamm- und Prozessentwicklung, das heißt im Labormaßstab, die Ausnahme. Wir präsentieren in dieser Studie eine integrative Analyse eines „Seperate Hydrolysis and Co-Fermentation (SHCF)"-Prozesses zur Bioethanol-Herstellung aus Weizenstroh. Der Prozess beinhaltet die Herstellung der Zellulasen und Hemizellulasen durch die Kultivierung einer *T. reesei*-Mutante auf vorbehandelte, Weizenstroh und die effiziente Co-Fermentation von Glukose und Xylose mit dem *S. cerevisiae*-Stamm IBB10B05. Die Prozesskonfigurationen wurden variiert und mit Hinsicht auf den Ethanol-Ertrag des gesamten Prozesses evaluiert. Das dadurch entstandene Verständnis für den Prozess ermöglichte es jene Prozessparameter herauszufinden, welche den stärksten Einfluss auf die Prozesseffizienz ausüben. Die höchste Prozessausbeute – 71.2 g Ethanol pro kg unbehandeltem Weizenstroh – war dabei

vergleichbar mit der Effizienz von Pilotanlagen. Prozessanalyse im Labormaßstab stellt daher ein sinnvolles Instrument zur Prozessoptimierung da.

Die in dieser Arbeit präsentierten Strategien zur Stamm- und Prozessentwicklung tragen zur Optimierung der kritischen Schritte im Bioraffinerieprozess bei. Die erzielten Verbesserungen von Schlüsselparametern sind ein wichtiger Schritt, um dem Ziel einer großtechnischen Verwertung von Lignozellulose als erneuerbarem Rohstoff entgegenzugehen.

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Chapter 1

Elucidation of the micromorphology of *Trichoderma reesei* mutant strains in wheat straw and lactose cultivations and its relationship to enzyme production

Elucidation of the micromorphology of *Trichoderma reesei* mutant strains in wheat straw and lactose cultivations and its relationship to enzyme production

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Abstract

Background

Trichoderma reesei is the principal producer of cellulolytic enzymes. Because of the strong influence on the enzyme production, the morphology of the filamentous fungi is a key parameter for process optimization. For efficient and cost-effective production of cellulolytic enzymes, cultivation of *T. reesei* is performed on lignocellulosic waste streams. These insoluble substrates prevent the application of conventional light microscopy for the analysis of fungal morphology. In this study we present a novel method for micromorphological analysis based on confocal laser scanning microscopy (CLSM) and computer-aided image analysis. This method enabled the quantification of the dimensions of the single cell (intercalary length and cell width) and the degree of branching in cultivations on the industrially relevant substrates wheat straw and lactose. The micromorphology of 2 *T. reesei* strains, QM9414 and a carbon catabolite derepressed *cre1* knockout mutant (Δcre), was analyzed in dependence of substrate, inoculation method, and agitation velocity.

Results

T. reesei strain Δcre formed shorter cells (10.09 µm) on average and developed more ramified mycelia (0.36 branches/cell) than strain QM9414 (12.03 µm, 0.22 branches/cell). Cultivated on wheat straw, the average cell length of QM9414 (10.87 µm) and Δcre (9.74 µm) was 10% and 26% shorter as compared to reference cultivations on lactose. When inoculation was done with spores as compared to hyphal biomass, the cell lengths of QM9414 (10.97 µm) and Δcre (9.10 µm) were on average about 20% shorter. Strain performance was evaluated in protein concentration

and total cellulase activity, which varied between 0.69 and 2.31 FPU mL^{-1} for Δcre and between 0.84 and 1.64 FPU mL^{-1} for QM9414. The cell length negatively correlated with the protein (regression coefficient -0.04 g L^{-1} μm^{-1}, R^2 0.33) and the cellulase (-0.30 FPU mL^{-1} μm^{-1}, R^2 0.53) production.

Conclusions

The dimensions of the single cell of *T. reesei* were dependent on the strain background, the substrate used and the process conditions applied. Micromorphological changes were correlated semi-quantitatively with the efficiency of enzyme production. In providing an analytical tool for process analysis for enzyme production by *T. reesei* on lignocellulosic substrate, this study has relevance for characterization and optimization of a key critical step in the overall lignocellulose saccharification process.

Keywords: *Trichoderma reesei*, morphology, cellulase production, lignocellulose, wheat straw, lactose

1. Background

The commercial viability of biorefinery processes is heavily dependent on the costs of cellulolytic enzymes [1]. To successfully utilize lignocellulose as carbon source, it is therefore necessary to intensify the enzyme production process [1, 2]. Based on the fast biomass growth and the ability to secrete high amounts of proteins, the filamentous fungi *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) has become the principal producer of cellulolytic enzymes [3]. However, the complex morphology of the fungus presents a major challenge in process development [4-6]. *T. reesei,* like other filamentous fungi such as *Aspergillus spp.,* can develop into various micro- and macromorphologic states [4-6]. The micromorphology describes the dimensions of the cells and the hyphae, as well as the degree of branching and the total number of tips [4, 6]. The macromorphology of the fungi can be broadly classified into pellets and freely dispersed mycelia [5, 6]. By employing wide-field light microscopy, it has been shown that the micro- and the macromorphology of cellulase-producing *T. reesei* is dependent on the carbon source [7-9], the composition of the culture medium [7, 8], the pH [10], the size of the inoculum [8], and the intensity of agitation [7, 11]. With the aim of quantifying morphological changes, the acquired images were analyzed towards the projected area of free and entangled mycelia, as well as the dimensions of the hyphae and the level of branching [7, 11-13]. This enabled the correlation of the micromorphology with the cellulase productivity [7, 11, 12], and proved that the micromorphology is a key factor for process analysis and optimization. However, there are limitations with this approach. Firstly, efficient and cost-effective production of enzymes often requires the cultivations of *T. reesei* on hemicellulosic and cellulosic waste streams with high solid loadings [14]. These substrates are insoluble

and often bulky or fibrous, and preclude the application of wide-field light microscopy. Consequently, studies have been exclusively conducted on model substrates, e.g. avicel or solca flocs [7-9, 12, 13, 15, 16]. Secondly, the fungal hyphae form filamentous 3-dimensional networks. Wide-field light microscopy, however, only provides images representing a 2-dimensional projection of this network. Only a thin section of the sample, corresponding to the depth of focus, is imaged sharply; the regions above and below this section are unsharp. This complicates quantitative image analysis. Furthermore, as all structures are projections into the image plane no axial distances can be measured.

In this study we present a new quantitative method for the analysis of fungal micromorphology. By using confocal laser scanning microscopy (CLSM) and computer-aided image analysis, it was possible to analyze and quantify the dimensions of the single cells and the degree of branching in cultivations on wheat straw and lactose. Based on its abundance and low price, wheat straw constitutes a promising renewable carbon source in Europe [10]. Lactose also induces expression of the complete set of hemicellulases and cellulases in *T. reesei* [17], although the level of expression is much lower as compared to wheat straw [18]. Lactose is the preferred carbon source when a soluble substrate is required [19]. The micromorphology was analyzed in 2 different *T. reesei* strains; the reference strain QM9414 [20] and strain Δcre. The latter has the *cre1* transcription factor removed from its genome and is therefore incapable of carbon catabolite repression. Based on the higher cellulase productivity, *cre1* knockout mutants are the preferred strains for efficient enzyme production [20-23]. By applying the developed CLSM-based method the dimensions of the single cells were analyzed in dependence of strain, carbon

source, inoculation method, and agitation velocity. The micromorphological changes were then correlated with the protein secretion and the enzyme production.

2. Results and Discussion

2.1. Macromorphology of strains QM9414 and Δcre

When *T. reesei* strains QM9414 and Δcre were cultivated on lactose, a distinct difference in the macromorphology was observed (Additional file 1). Whereas Δcre developed into small and dense pellets, QM9414 formed larger and loose pellets (Additional file 1). On wheat straw both strains showed dispersed growth. The straw fibers likely prevented the aggregation of hyphae and spores, which is the initial step in pellet formation [24]. A similar effect has been described for the addition of micro-particles to cultivations by filamentous fungi [25, 26]. As expected, the high solid content of the cultivations precluded analysis of fungal morphology in wheat straw cultivations by light microscopy (Additional file 1).

2.2. Analysis of fungal micromorphology by CLSM

QM9414 and Δcre were cultivated on wheat straw and lactose. Samples were taken after 2 and 8 days of cultivations and analyzed by CLSM. An example of the resulting images is depicted in Additional file 2. All images were of high resolution, and hyphae walls, branching points, and the septa (which divide the hyphae into cells) were clearly visible (Additional file 2). In cultivations on wheat straw, it was possible to distinguish the wheat straw fibers from the fungal mycelia (Additional file 3). Samples were also taken and analyzed after 14 days of incubation. An example of the resulting CLSM image and the comparison to the respective 8-day sample is depicted in Additional file 4. The images lost contrast with incubation time, leading to a lower

image quality and resolution. The probable reason for this was the induction of autophagy [27] and the resulting accumulation of cell wall fragments and the unspecific straining thereof.

2.3. Protein production and biomass growth

The protein concentrations were analyzed over time in lactose and wheat straw cultivations by *T. reesei* strains QM9414 and Δcre (Additional file 5). Regardless of the cultivation conditions, both strains showed a lag phase of approximately 50 h. Afterwards the protein concentrations started to increase until it plateaued at approximately ~200 h of cultivation. After ~250 h the protein concentrations increased again. The final increase was probably caused by cell proteins being liberated by autophagy [27].

As exemplified by the CLSM images in Additional file 2, both strains formed long unbranched hyphae after 2 days and developed into a highly ramified and interwoven hyphae network after 8 days of incubation (Additional file 2).

The protein time courses and the observed changes in micromorphology were comparable to the model described in Velkovska et al. (1997). The authors suggested that biomass growth in *T. reesei* proceeds in 2 phases, resulting in a moderately branched primary mycelium and highly ramified secondary mycelium, where only the secondary mycelium actively secretes enzymes [28].

Biomass growth of strains QM9414 and Δcre was further analyzed in lactose cultivations inoculated with spores and a piece of overgrown agar (Additional file 5). After 8 days of cultivations, the wet weight of Δcre was approximately 1.3-fold higher as compared to QM9414. Further, both strains produced 1.7-fold more biomass when

cultivations were inoculated with a piece of overgrown agar than with spores (Additional file 5).

2.4. CLSM image acquisition and analysis for quantification of fungal micromorphology

CLSM features many advantages over wide-field microscopy, which are essential for the analysis and accurate quantification of the fungal micromorphology in cultivations on insoluble and soluble substrates. Firstly, based on the specific pattern of the staining and the resulting fluorescence signal, structural differences between the fungal biomass and the plant material can be discerned. This made it possible to differentiate the hyphae from the wheat straw fibers, which would not be possible in conventional light microscope images (Additional file 3). Secondly, CLSM yields an image of a thin sample section coinciding with the objective focal plane, without unfocused contributions from other regions of the sample. The absence of out-of-focus light results in a higher contrast and sharper images, facilitating quantitative analysis. Thirdly, by recording images at a range of axial (z) positions, a set of xy images is obtained which contains full 3-dimensional information (xyz). This set of images ("z-stack") allows length measurements of cells oriented not only horizontally, but also at any angle with respect to the focal plane. In contrast to wide-field light microcopy studies, where only the projected hyphal dimensions could be measured [7, 11-13, 15, 29], CLSM therefore enables the quantification of the *actual* dimensions of the cells.

A MATLAB program has been developed for quantification of the geometry of the fungal network from the CLSM images. The program interactively displays the images of different z sections and allows the user to identify the fungal cells in 3-

dimensions. Based on the septa positions identified by the user and on the fluorescence intensity of the cell walls, the program automatically calculates the intercalary length and the cell width, respectively. This semiautomated approach facilitated highly accurate length and width measurements. An example of the interface and the 3-dimensional hyphae skeleton is given in Additional file 6.

2.5. Analysis and quantification of fungal micromorphology in dependence of strain, carbon source and cultivation conditions and its correlation to enzyme production

Because of the observed differences in biomass growth between the strains and the inoculation methods (Additional file 5), precultures were performed in glucose media to equalize the starting conditions. Results presented below are from the main cultures, which were inoculated from the precultures. Samples for analysis of the micromorphology and enzyme production were taken after 8 days of incubation, when the protein concentration was highest and before induction of autophagy (Additional file 4 and 5). Table 1 summarizes the cell lengths and widths, the numbers of branches formed per cell, the protein concentrations and the volumetric cellulase activities from the presented cultivation conditions.

2.5.1. *T. reesei* strains QM9414 and Δcre

When cultivated on wheat straw, *T. reesei* strains QM9414 and Δcre produced between 0.69 and 2.31 FPU mL^{-1} in 8 days (Table 1). Both strains showed significant variations in the secreted total cellulase activity. Under the same cultivation conditions Δcre always produced a higher volumetric cellulase activity than QM9414 (Table 1). Because QM9414, in contrast to Δcre, is carbon catabolite repressed this result was as expected [20-23]. On lactose, both strains only produced basal amounts

of cellulases, and the resulting volumetric activity did not reach the lower detection limit of the FPU assay. Therefore the total protein concentration was measured in the cultivation supernatant. Within the different experimental setups no significant variations between strain QM9414 and Δcre could be observed (Table 1),

The CLSM images depicted in Figure 1 and 2 show distinct micromorphological differences between strains QM9414 and Δcre. To enable comparison of the 2 strains, the average cell length, cell width and number of branches per septa were calculated for wheat straw and lactose cultivations, respectively. The results are summarized in Table 2. Independent of the carbon source, Δcre formed shorter and wider cells than QM9414. Δcre further developed a more ramified mycelia with up to 2.25-fold more branches per cell (Table 2). In filamentous fungi, glucose is a precursor for the major cell wall components chitin and glucan [30, 31]. It therefore seems likely that manipulation of glucose catabolism does not only influence biomass growth and protein productivity but also the fungal morphology. Removal or mutation of the *cre1* transcription factor in *T. reesei* has been shown to result in severe distortion of colony morphology [22]. Similar to observations made in this study, removal of the *cre1* analog *creA* also resulted in an increased degree of branching in submerged cultures by *A. niger* [31].

2.5.2. Wheat straw and lactose cultivations

In cultivations on lactose both strains produced 50% less proteins on average than on wheat straw (Table 1, Additional file 2), and only basal amounts of filter paper activity were detected. This was in accordance with proteome studies, where it has been shown that although lactose induces expression of the complete set of

hemicellulases and cellulases [17], the level of expression is much lower as compared to wheat straw [18].

In cultivations on wheat straw, strains QM9414 and Δcre developed 9% and 27% shorter cells on average as compared to lactose cultivations. The cells of Δcre were further 12% wider (Table 1 and 2). QM9414 also tended to form wider cells on wheat straw (Table 1). However, the changes were less pronounced.

When cultivated on cellulase-inducing substrates, *T. reesei* has been shown to develop an extensive extracellular fibrous layer [16]. This layer consists of a heteroglycan, which captures and activates hemicellulolytic and cellulolytic enzymes [32-34]. Because wheat straw induces cellulase production much stronger than lactose does [18], it seems likely that the increase in cell diameter was at least partially caused by a more extensive development of this heteroglycan layer.

In an early work on *Geotrichum candidum* it has been shown that the length of the non-apical cells is dependent on the glucose concentration [35]. An increase from 0 to 10 g/L resulted in an up to 2-fold increase in the intercalary length [35]. In cultivations on wheat straw by *T. reesei*, the availability of sugar monomers is dependent on the rate of cellulose and hemicellulose hydrolysis by the secreted enzymes. It therefore seems likely that the concentration of free sugars in cultivations on wheat straw is much lower as compared to lactose cultivations. In accordance to observations made for *G. candidum* [35], this might explain the decreased cell length in wheat straw as compared to lactose cultivations.

2.5.3. Inoculation method

Cultivations were either started by adding a piece of overgrown agar or by adding spores to a final concentration of 10^5 spores per mL. With the piece of overgrown

agar, preexisting hyphal biomass was added to the cultivations. Because the fungus does not have to develop from the spores, this method results in faster onset of biomass formation. Consequently, 1.7-fold more biomass was obtained in agar- as compared to spore-inoculated cultivations (Additional file 3). In literature, however, inoculation with spores has been the method of choice [8, 36-38]. To analyze the impact on the micromorphology, the cell length was compared in dependence of the inoculation method and the results are depicted in Figure 3 (panel A1 and A2). Independent of substrate, agitation velocity and strain, agar-inoculated cultivations resulted in longer septa as compared to spore-inoculated cultures (Figure 3, panel A1 and A2). It is noteworthy that the micromorphological analysis was conducted from samples of the main cultures. Thus, the shift in micromorphology caused by the inoculation method was sustained over the precultures and was still detectable in the main cultivations.

The inoculation method further affected the degree of branching (Figure 1 and 2), as well as the cellulase production (Table 1). Thus, in spore-inoculated cultivations, QM9414 and Δcre formed averagely 5% and 8% more branches per cell as compared to agar-inoculated cultivations. When wheat straw cultivations were inoculated with spores, QM9414 and Δcre produced 1.4 FPU mL^{-1} and 2 FPU mL^{-1} on average (Table 1). This is 1.7-fold and 1.4-fold higher as compared to agar-inoculated cultivations by QM9414 (0.8 FPU mL^{-1}) and Δcre (1.4 FPU mL^{-1}), respectively. Protein secretion has been suggested to primarily happen at the tip of the apical compartment [39, 40]. The higher degree of branching and the corresponding increase in freshly formed tips therefore likely caused the observed increase in enzyme production.

2.5.4. Agitation velocity

Because of its impact on oxygen input, morphology and fragmentation, the type and intensity of agitation is a key parameter for cultivations by filamentous fungi [11, 12, 41-45]. To analyze the impact of the agitation velocity on the micromorphology of strains QM9414 and Δcre, cultivations were incubated at 190 rpm and 150 rpm. The resulting cell lengths are compared in Figure 3 (panel B1 and B2). In cultivations on wheat straw no clear effect of the agitation velocity on the cell length was observed. On lactose, however, incubation at 190 rpm seemingly resulted in longer cells as compared to 150 rpm (Figure 3).

It has been shown that the length of the apical compartment increases with increasing biomass growth rates [46]. The longer cells observed at 190 rpm might therefore be a result of the increased oxygen transfer rate and the resulting higher biomass growth. An increase in the intercalary cell length with higher stirring rates has been also described for *A. nidulans* strains [47].

Wheat straw cultivations showed a much higher viscosity because of the solids load and the dispersed macromorphology (Additional file 1). The difference between 150 rpm and 190 rpm was therefore probably not enough to influence the oxygen transfer rate to the extent necessary to induce changes in the cell length (Figure 3).

2.5.5. Tween80

The influence of the surface-active agent Tween80 on the micromorphology and enzyme production was analyzed in wheat straw cultivations. In agar-inoculated cultivations, both strains tended to develop longer cells and a lower filter paper activity when Tween80 was added to the culture media (Table 1). In spore-inoculated cultivations, Δcre developed shorter cells and produced more FPU mL^{-1} when

Tween80 was added. QM9414 developed longer cells in spore-inoculated cultivations with supplemented Tween80, and a higher cellulase activity (Table 1). It has been described that Tween80 increases the permeability of the cell membrane. This facilitates protein secretion, and thus positively influences the cellulase production by *T. reesei* [7, 8, 48]. In this study, however, no clear effect of Tween80 on the micromorphology or the enzyme production could be observed (Table 1).

2.6. Micromorphological changes at single cell level affect the protein and cellulase production

As presented above, the dimensions of the cells were varying in dependence of strain, substrate and process conditions. In Figure 4 (panel A) the cell length and the total cellulase activity (FPU mL^{-1}) of all presented wheat straw cultivations are compared. The results were sorted according to the measured cell lengths, and it appeared as both strains tended to produce more cellulases in cultivations when shorter cells were formed. The cellulase activity (FPU mL^{-1}) was also plotted over the corresponding cell length and the regressed change in filter paper activity with increasing cell length was -0.30 FPU mL^{-1} μm^{-1} (R^2 0.53). To include lactose cultivations, where low protein expression precluded measurement of the filter paper activity, the protein concentration of all experimental setups was plotted against the cell length. The results are depicted in Figure 4 (panel B). Regardless of strain, carbon source, agitation velocity or inoculation method used, the protein concentrations were higher in cultivations that elicited shorter cells. The protein concentration correlates with the cell length with a regression coefficient of -0.04 g L^{-1} μm^{-1} (R^2 0.33). This was in accordance with the observed trend for cultivations on wheat straw (Figure 1, panel

A), and it can be concluded that there was a negative correlation between the cell length and the protein secretion.

Filamentous fungi grow highly polarized by extension of the apical compartment [6]. The extension proceeds until the cell is through an active mitotic cell cycle in which the multiple nuclei duplicate, and stops with the septation of the compartment [46, 49]. The size of the apical compartment is thereby variable and dependent on the maximal growth rate, where faster growth is linked to a longer apical cell [46]. It has been further observed that, with an increase in biomass growth, the hyphae diameter is also increasing [46, 47, 50]. A higher biomass growth rate, in turn, can result in higher protein productivities [50]. A positive correlation between cellulase productivity and hyphae diameter has been described for cultivations by *T. reesei* [7]. In this study, a decrease in cell lengths was accompanied by an increase in cell diameter (Figure 5, regression coefficient -0.11 $\mu m\ \mu m^{-1}$, R^2 0.42). The shorter, and consequently wider cells, observed in cultivations where cellulase production was high (Figure 4) might therefore indicate that the fungal strains grew at faster rates, which resulted in increased protein productivities [7, 50]. It has been further shown that the branching mainly occurs at the first subapical compartment [40]. The shorter cells and the corresponding faster septation might therefore have induced the formation of more branches [40]. A higher degree of branching that is accompanied by an increase in freshly formed hyphae tips has been described to increase protein secretion [39, 40].

3. Conclusions

In this study, we present for the first time the analysis and quantification of the micromorphology of *T. reesei* growing on a solid lignocellulosic substrate. CLSM

image acquisition and computer-aided image analysis enabled the quantification of the dimensions of single cells and the degree of branching in cultivations on wheat straw and lactose. The micromorphology was strongly dependent on strain, carbon source and inoculation method. Changes in cell length were correlated with the protein (0.04 mg mL^{-1} μm^{-1}; R^2 0.33) and cellulase production (-0.30 FPU mL^{-1} μm^{-1}, R^2 0.53). This study shows for the first time that the micromorphology at single cell level affects the enzyme productivity in *T. reesei*. The herein presented method therefore provides a useful tool to analyze and optimize cultivations of *T. reseei* on lignocellulosic feedstocks.

4. Methods

4.1. Chemicals and media

Unless stated otherwise, all chemicals were from Carl Roth + Co KG (Karlsruhe, Germany). Potato-dextrose-agar (PDA) plates were prepared as described by the manufacturer. Mineral media (MM) contained 5 g/L yeast extract, 5 g/L KH_2PO_4, 3.75 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4 \times 7H_2O$, 0.3 g/L $CaCl_2 \times 2H_2O$, 100 µL/L Antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA), 0.5 g/L rapeseed oil and 1 mL/L trace elements (5 g/L $FeSO_4 \times 7H_2O$, 1.6 g/L $MnSO_4 \times H_2O$, 1.4 g/L $ZnSO_4 \times 7H_2O$, 2 g/L $CoCl_2 \times 6H_2O$, 15 g/L EDTA disodium chloride salt). Tween80 supplemented cultivations additionally contained 0.1 % (w/w) Tween80. As carbon source 10 g/L glucose, 14 g/L lactose or 14 g dry mass/L wheat straw was used as indicated. The wheat straw was pretreated by steam explosion. A detailed description of the pretreatment method and the composition of the feedstock can be found elsewhere [2].

4.2. Strains and maintenance

The *T. reesei* mutant strains QM9414 and Δcre were utilized. Both strains were kindly provided by Bernhardt Seiboth (Vienna University of Technology, Wien, Austria) and stored as spores in 15% glycerol at -80°C.

4.3. Shaken flak cultivations

All cultivations were conducted in 300 mL wide mouth Erlenmeyer flasks closed with cotton wool stoppers. Incubation was at 28°C and 190 rpm or 150 rpm in a Certomat BS-1 orbital incubator shaker (Sartorius AG, Göttingen, Germany). 2 different inoculation methods were applied in this study, either with a piece of overgrown agar or with spores. For the former, 100 µL of glycerol stock was streaked on PDA plates and incubated at 30°C for 3 days. Subsequently a piece (~ 2 cm^2) of overgrown agar was cut out and transferred to the cultivation medium. For the inoculation with spores, PDA plates were incubated until sporulation occurred (12 to 24 days). Spores were then harvested by washing the plates with a Triton X-100 solution (0.1 % (w/v), Sigma). The spore concentration was determined with a Neubauer counting chamber. The spore solution was added to the cultivation medium to final concentration of 10^5 spores per mL. Cultivations were either inoculated directly or from a preculture. Precultures were performed in glucose-based MM. Inoculation was with the 2 methods described above. Incubation of the precultures was at 28°C and 190 rpm (Certomat) for 48 h. The biomass was harvested by centrifugation (15,700g, 4°C for 10 min, Eppendorf 5415 R, Eppendorf, Hamburg, Germany) and 40 mg wet weight of biomass was used to inoculate the main cultivation.

4.4. Sampling, determination of protein concentration and cellulase activity

Samples were taken for analysis of micromorphology and enzyme production. For image analysis the fungal mycelia was fixated with 10 % (v/v) formaldehyde solution (100 g/L) and stored at 4°C. For analysis of total cellulase activity (FPU mL^{-1}) and protein concentration, samples were centrifuged (15,700 g, 4°C for 10 min, Eppendorf 5415 R, Eppendorf, Hamburg, Germany) and the supernatant stored at 4°C. For determination of protein concentration, proteins were precipitated and quantified against a BSA standard utilizing the Roti-Quant kit (Roth) and following the manufacturer's instructions. FPU activity was measured following International Union of Pure and Applied Chemistry (IUPAC) recommendations [51].

4.5. CLSM image acquisition and analysis

For CLSM analysis the samples were prepared as follows. The fixated biomass was washed with 50 mM sodium carbonate-bicarbonate buffer (pH 9.2) and then transferred to an object slide. The samples were immersed with a Calcoflour White solution (0.01 g/L, Sigma) and incubated for 5 min. The samples were imaged on a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Wetzlar Germany) using a 40x/1.15 oil objective. The excitation wavelength was 405 nm, the emission was detected in the spectral range 420-530 nm, matching the spectral characteristics of the dye Calcofluor White. Typically, a series of 60-100 confocal images at different axial positions (z-stack) was recorded, yielding a 3-dimensional representation of the sample. The spacing between neighboring images in the axial direction (z) was 0.46 µm, resulting in the total imaged volume depth of 28-46 µm. The lateral size of the images was 183×183 µm (1024×1024 pixels). An interactive program with a graphical user interface was written in MATLAB (MathWorks,

Natick, MA, USA) to analyze the morphology of the fungi from the 3-dimensional confocal images. The software displays the images and allows scrolling through the 3rd (axial) dimension. The user is required to identify the center positions of the septa between the cells by a mouse click, to connect the pairs of septa defining a cell, and to mark branching cells. In this way, a 3-dimensional skeleton of the fungal hyphae is defined. The software then automatically calculates the cell lengths and, on basis of the fluorescence intensity of cell walls, the cell widths. The results are saved in a text file for further analysis. An example of the interface is given in Additional file 6. Examples of the cell length distribution are depicted in Additional file 7.

Authors' contributions

VN and BN designed the research. VN and MS planned and performed the experiments. VN, MS, ZP and ME analyzed and processed the data. The manuscript was written from contributions of all authors. VN and BN wrote the paper. All authors have read and approved the final manuscript.

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Competing interest

The authors declare that they have no competing interests

References

1. Sassner P, Galbe M, Zacchi G: **Techno-economic evaluation of bioethanol production from three different lignocellulosic materials.** *Biomass Bioenergy* 2008, **32:**422-430.
2. Novy V, Longus K, Nidetzky B: **From wheat straw to bioethanol: integrative analysis of a separate hydrolysis and co-fermentation process with implemented enzyme production.** *Biotechnol Biofuels* 2015, **8:**46.
3. Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, et al: **Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*).** *Nat Biotechnol* 2008, **26:**553-560.
4. Grimm LH, Kelly S, Krull R, Hempel DC: **Morphology and productivity of filamentous fungi.** *Appl Microbiol Biotechnol* 2005, **69:**375-384.
5. Krull R, Wucherpfennig T, Esfandabadi ME, Walisko R, Melzer G, Hempel DC, Kampen I, Kwade A, Wittmann C: **Characterization and control of fungal morphology for improved production performance in biotechnology.** *J Biotechnol* 2013, **163:**112-123.
6. Wucherpfennig T, Kiep KA, Driouch H, Wittmann C, Krull R: **Morphology and rheology in filamentous cultivations.** In *Adv Appl Microbiol. Volume* Volume 72. Edited by Allen I. Laskin SS, Geoffrey MG: Academic Press; 2010: 89-136.
7. Ahamed A, Vermette P: **Effect of culture medium composition on *Trichoderma reesei*'s morphology and cellulase production.** *Bioresour Technol* 2009, **100:**5979-5987.

8. Domingues FC, Queiroz JA, Carbral JMS, Fonseca LP: **The influence of culture conidtions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30.** *Enzyme Microb Technol* 2000, **26:**394-401.

9. Peciulyte A, Anasontzis GE, Karlstrom K, Larsson PT, Olsson L: **Morphology and enzyme production of *Trichoderma reesei* Rut C-30 are affected by the physical and structural characteristics of cellulosic substrates.** *Fungal Genet Biol* 2014.

10. Govumoni SP, Koti S, Kothagouni SY, S V, Linga VR: **Evaluation of pretreatment methods for enzymatic saccharification of wheat straw for bioethanol production.** *Carbohydr Polym* 2013, **91:**646-650.

11. Patel N, Choy V, Malouf P, Thibault J: **Growth of *Trichoderma reesei* RUT C-30 in stirred tank and reciprocating plate bioreactors.** *Process Biochem* 2009, **44:**1164-1171.

12. Ahamed A, Vermette P: **Effect of mechanical agitation on the production of cellulases by *Trichoderma reesei* RUT-C30 in a draft-tube airlift bioreactor.** *Biochem Eng J* 2010, **49:**379-387.

13. Lecault V, Patel N, Thibault J: **Mophological charcterization and viability assessment of *Trichoderma reesei* by image analysis.** *Biotechnol´ Prog* 2007, **23:**734-740.

14. Aro N, Pakula T, Penttila M: **Transcriptional regulation of plant cell wall degradation by filamentous fungi.** *FEMS Microbiol Rev* 2005, **29:**719-739.

15. Li C, Yang Z, Zhang RH, Zhang D, Chen S, Ma L: **Effect of pH on cellulase production and morphology of *Trichoderma reesei* and the application in cellulosic material hydrolysis.** *J Biotechnol* 2013, **168:**470-477.

16. Nitta M, Shida Y, Okada H, Osumi M, Ogasawara W: **Hyphal surface architecture and cell morphology of *Trichoderma reesei*.** *J Electron Microsc (Tokyo)* 2012, **61:**187-192.

17. Ivanova C, Baath JA, Seiboth B, Kubicek CP: **Systems analysis of lactose metabolism in *Trichoderma reesei* identifies a lactose permease that is essential for cellulase induction.** *PLoS One* 2013, **8:**e62631.

18. Bischof R, Fourtis L, Limbeck A, Gamauf C, Seiboth B, Kubicek CP: **Comparative analysis of the *Trichoderma reesei* transcriptome during growth on the cellulase inducing substrates wheat straw and lactose.** *Biotechnol Biofuels* 2013, **6:**127.

19. Olsson L, Christensen TMIE, Hansen KP, Palmqvist EA: **Influence of the carbon source on production of cellulases, hemicellulases and pectinases by *Trichoderma reese*i Rut C-30.** *Enzyme Microb Technol* 2003, **33:**612-619.

20. Peterson R, Nevalainen H: ***Trichoderma reesei* RUT-C30--thirty years of strain improvement.** *Microbiology* 2012, **158:**58-68.

21. Ilmen M, Thrane C, Penttila M: **The glucose repressor gene cre1 of *Trichoderma: isolation* and expression of a full-length and a truncated mutant form.** *Mol Gen Genet* 1996, **251:**451-460.

22. Nakari-Setala T, Paloheimo M, Kallio J, Vehmaanpera J, Penttila M, Saloheimo M: **Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production.** *Appl Environ Microbiol* 2009, **75:**4853-4860.

23. Portnoy T, Margeot A, Linke R, Atanasova L, Fekete E, Sandor E, Hartl L, Karaffa L, Druzhinina IS, Seiboth B, et al: **The CRE1 carbon catabolite**

repressor of the fungus *Trichoderma reesei*: a master regulator of carbon assimilation. *BMC Genomics* 2011, **12:**269.

24. Nielsen J: **Modelling the morphology of filamentous microorganisms.** *Trends Biotechnol* 1996, **14:**438-443.

25. Kaup BA, Ehrich K, Pescheck M, Schrader J: **Microparticle-enhanced cultivation of filamentous microorganisms: increased chloroperoxidase formation by *Caldariomyces fumago* as an example.** *Biotechnol Bioeng* 2008, **99:**491-498.

26. Etschmann MM, Huth I, Walisko R, Schuster J, Krull R, Holtmann D, Wittmann C, Schrader J: **Improving 2-phenylethanol and 6-pentyl-alpha-pyrone production with fungi by microparticle-enhanced cultivation (MPEC).** *Yeast* 2015, **32:**145-157.

27. Pollack JK, Harris SD, Marten MR: **Autophagy in filamentous fungi.** *Fungal Genet Biol* 2009, **46:**1-8.

28. Velkovska S, Marten MR, Ollis DF: **Kinetic model for batch cellulase production by *Trichoderma reesei* RUT c30.** *J Biotechnol* 1997, **54:**83-94.

29. Lecault V, Patel N, Thibault J: **An image analysis technique to estimate the cell density and biomass concentration of Trichoderma reesei.** *Lett Appl Microbiol* 2009, **48:**402-407.

30. Wessels JGH: **Cell wall synthesis in apical hyphal growth.** In *International Review of Cytology. Volume* Volume 104. Edited by Bourne GH: Academic Press; 1986: 37-79.

31. Agger T, Petersen JB, O'Connor SM, Murphy RL, Kelly JM, Nielsen J: **Physiological characterisation of recombinant *Aspergillus nidulans* strains**

with different *creA* genotypes expressing *A. oryzae* alpha-amylase. *J Biotechnol* 2002, **92:**279-285.

32. Messner R, Hagspiel K, Kubicek CP: **Isolation of a β-glucosidase binding and activating polysaccharide from cell walls of *Trichoderma reesei*.** *Arch Microbiol*, **154:**150-155.

33. Sprey B: **Localisation of β-glucosidase in *Trichoderma reesei* cell walls with immunoelectron microscopy.** *FEMS Microbiol Lett* 1986, **36:**287-292.

34. Rath J, Messner R, Kosma P, Altmann F, Marz L, Kubicek CP: **The alpha-D-mannan core of a complex cell-wall heteroglycan of *Trichoderma reesei* is responsible for beta-glucosidase activation.** *Arch Microbiol* 1995, **164:**414-419.

35. Fiddy C, Trinci AP: **Kinetics and morphology of glucose-limited cultures of moulds grown in a chemostat and on solid media.** *Arch Microbiol* 1975, **103:**191-197.

36. Metz B, Kossen NWF: **The growth of molds in the form of pellets–a literature review.** *Biotechnol Bioeng* 1977, **19:**781-799.

37. El-Enshasy HA, Farid MA, El-Sayed E-SA: **Influence of inoculum type and cultivation conditions on natamycin production by *Streptomyces natalensis*.** *J Basic Microbiol* 2000, **40:**333-342.

38. Vecht-Lifshitz SE, Magdassi S, Braun S: **Pellet formation and cellular aggregation in *Streptomyces tendae*.** *Biotechnol Bioeng* 1990, **35:**890-896.

39. Peberdy JF: **Protein secretion in filamentous fungi — trying to understand a highly productive black box.** *Trends Biotechnol* 1994, **12:**50-57.

40. Müller C, McIntyre M, Hansen K, Nielsen J: **Metabolic engineering of the morphology of *Aspergillus oryzae* by altering chitin synthesis.** *Appl Environ Microbiol* 2002, **68:**1827-1836.

41. Amanullah A, Jüsten P, Davies A, Paul GC, Nienow AW, Thomas CR: **Agitation induced mycelial fragmentation of *Aspergillus oryzae* and *Penicillium chrysogenum*.** *Biochem Eng J* 2000, **5:**109-114.

42. Lejeune R, Baron GV: **Effect of agitation on growth and enzyme production of *Trichoderma reesei* in batch fermentation.** *Appl Microbiol Biotechnol*, **43:**249-258.

43. Marten MR, Velkovska S, Khan SA, Ollis DF: **Rheological, mass transfer, and mixing characterization of cellulase-producing *Trichoderma reesei* suspensions.** *Biotechnol Prog* 1996, **12:**602-611.

44. Mukataka S, Kobayashi N, Sato S, Takahashi J: **Variation in cellulase-constituting components from *Trichoderma reesei* with agitation intensity.** *Biotechnol Bioeng* 1988, **32:**760-763.

45. Amanullah A, Christensen LH, Hansen K, Nienow AW, Thomas CR: **Dependence of morphology on agitation intensity in fed-batch cultures of *Aspergillus oryzae* and its implications for recombinant protein production.** *Biotechnol Bioeng* 2002, **77:**815-826.

46. Müller C, Spohr AB, Nielsen J: **Role of substrate concentration in mitosis and hyphal extension of *Aspergillus*.** *Biotechnol Bioeng* 2000, **67:**390-397.

47. McIntyre M, Dynesen J, Nielsen J: **Morphological characterization of *Aspergillus nidulans*: growth, septation and fragmentation.** *Microbiology* 2001, **147:**239-246.

48. Reese ET, Maguire A: **Surfactants as stimulants of enzyme production by microorganisms.** *Appl Microbiol* 1969, **17:**242-245.

49. Clutterbuck AJ: **Synchronous nuclear division and septation in *Aspergillus nidulans*.** *J Gen Microbiol* 1970, **60:**133-135.

50. Agger T, Spohr AB, Carlsen M, Nielsen J: **Growth and product formation of *Aspergillus oryzae* during submerged cultivations: verification of a morphologically structured model using fluorescent probes.** *Biotechnol Bioeng* 1998, **57:**321-329.

51. Ghose T: **Measurement of cellulase activities.** *Pure Appl Chem* 1987, **2:**257 - 268.

Tables

Table 1 - Fungal micromorphology, protein concentration and cellulase activity obtained in cultivations on lactose and wheat straw by *T. reesei* strains Δcre and QM9414[a)]

	Cell length [µm]	Cell width [µm]	Branches/cell [-]	FPU mL^{-1}	Protein [g L^{-1}]
			T. reesei Δcre		
Wheat straw					
agar-190	10.21 ± 3.43	3.29 ± 1.34	0.40	1.74	0.59
agar-150	10.08 ± 3.17	2.80 ± 0.80	0.41	0.69	0.33
spore-190	8.82 ± 4.18	3.85 ± 0.89	0.38	1.88	0.57
spore-150	9.75 ± 2.92	3.47 ± 1.06	0.48	1.82	0.30
agar-190-Tw	11.73 ± 3.81	3.60 ± 1.51	0.40	1.27	0.38
spore-190-Tw	7.87 ± 2.21	3.98 ± 1.09	0.08	2.31	0.59
Lactose					
agar-190	11.75 ± 4.12	3.01 ± 0.77	0.43	n.d.	0.27
agar-150	11.67 ± 4.64	3.20 ± 0.56	0.25	n.d.	0.26
spore-190	10.51 ± 3.20	3.42 ± 0.97	0.28	n.d.	0.30
spore-150	8.55 ± 3.32	2.91 ± 1.01	0.50	n.d.	0.26
			T. reesei QM9414		
Wheat straw					
agar-190	11.84 ± 3.43	3.05 ± 0.94	0.05	0.88	0.64
agar-150	10.21 ± 2.92	3.04 ± 1.03	0.40	0.84	0.31
spore-190	10.42 ± 3.15	3.32 ± 1.23	0.01	1.45	0.58
spore-150	10.20 ± 3.21	2.35 ± 0.95	0.26	1.64	0.26
agar-190-Tw	12.47 ± 3.83	2.67 ± 1.27	0.06	0.77	0.43
spore-190-Tw	10.09 ± 2.67	3.30 ± 1.41	0.15	1.14	0.44
Lactose					
agar-190	16.74 ± 5.40	2.59 ± 0.69	0.25	n.d.	0.09
agar-150	14.24 ± 4.11	3.03 ± 1.20	0.31	n.d.	0.25
spore-190	13.53 ± 4.82	2.82 ± 0.64	0.23	n.d.	0.09
spore-150	10.59 ± 3.47	3.32 ± 0.53	0.49	n.d.	0.26

n.d. - not detectable

a) Volumetric cellulase activity (FPU mL^{-1}) and protein concentration represent mean values of 2 experiments. Dimensions of the cells represent mean values and the spread from 40 cells analyzed from 1 representative CLSM image. The branches per cell were analyzed in the same image and represent the value of 60 to 100 cells.

Table 2 - Average cell length and width and the number of branches per cell obtained in wheat straw and lactose cultivations by *T. reesei* strains Δcre and QM9414[a)]

	Cell length [μm]	Cell width [μm]	Branches/cell [-]
	T. reesei Δcre		
Wheat straw	9.74 ± 1.32	3.50 ± 0.42	0.36 ± 0.14
Lactose	10.62 ± 1.49	3.13 ± 0.23	0.36 ± 0.12
	T. reesei QM9414		
Wheat straw	10.87 ± 1.02	2.95 ± 0.38	0.16 ± 0.15
Lactose	13.77± 2.19	2.94 ± 0.31	0.32 ± 0.09

a) Data represent mean values and standard deviations. Data were taken from Table 1.

Figures

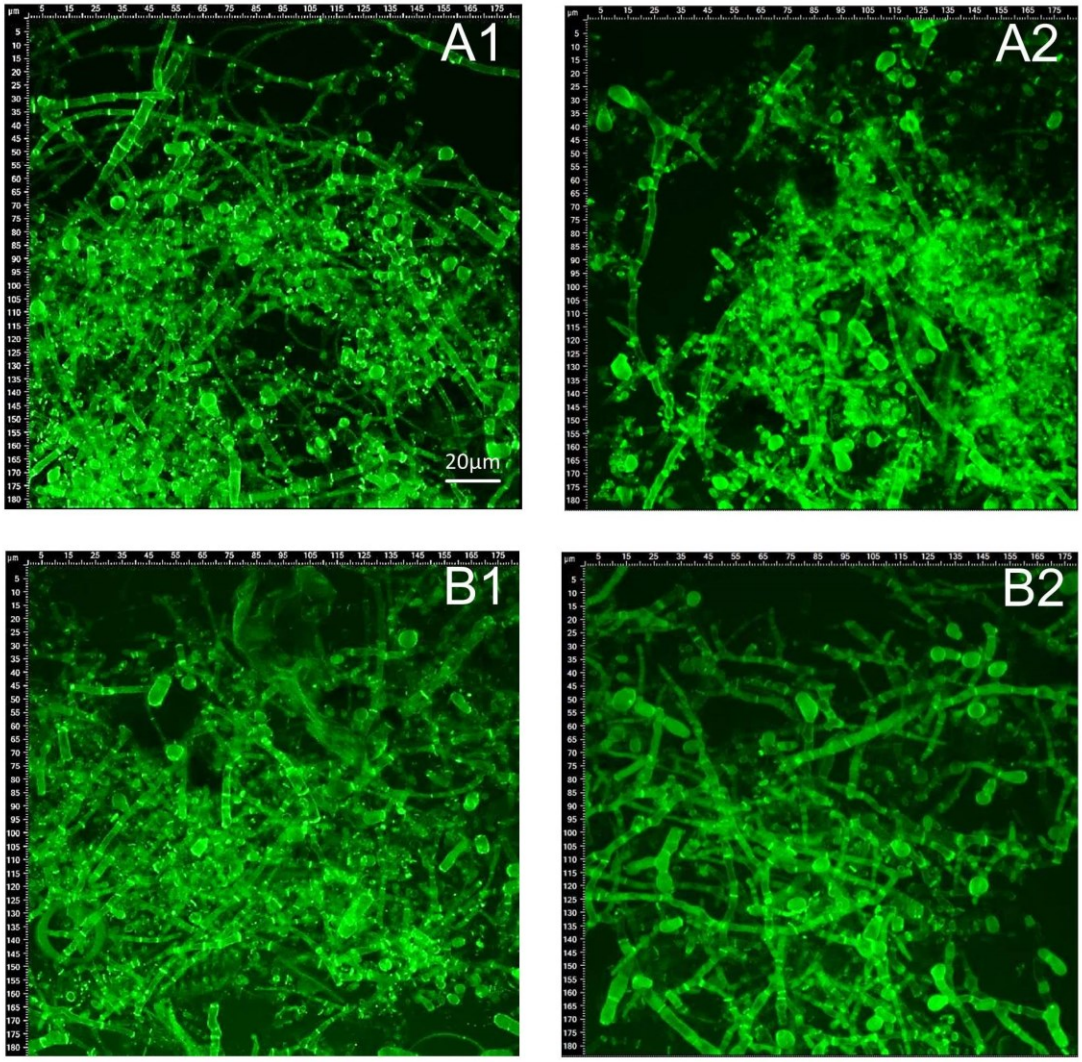


Figure 1 - CLSM images of wheat straw cultivations by *T. reesei* strains QM9414 (A1 and A2) and Δcre (B1 and B2)

Precultures were inoculated with 10^5 spores/mL (A1 and B1) or with a piece of overgrown agar (A2 and B2). All cultivations were incubated at 190 rpm.

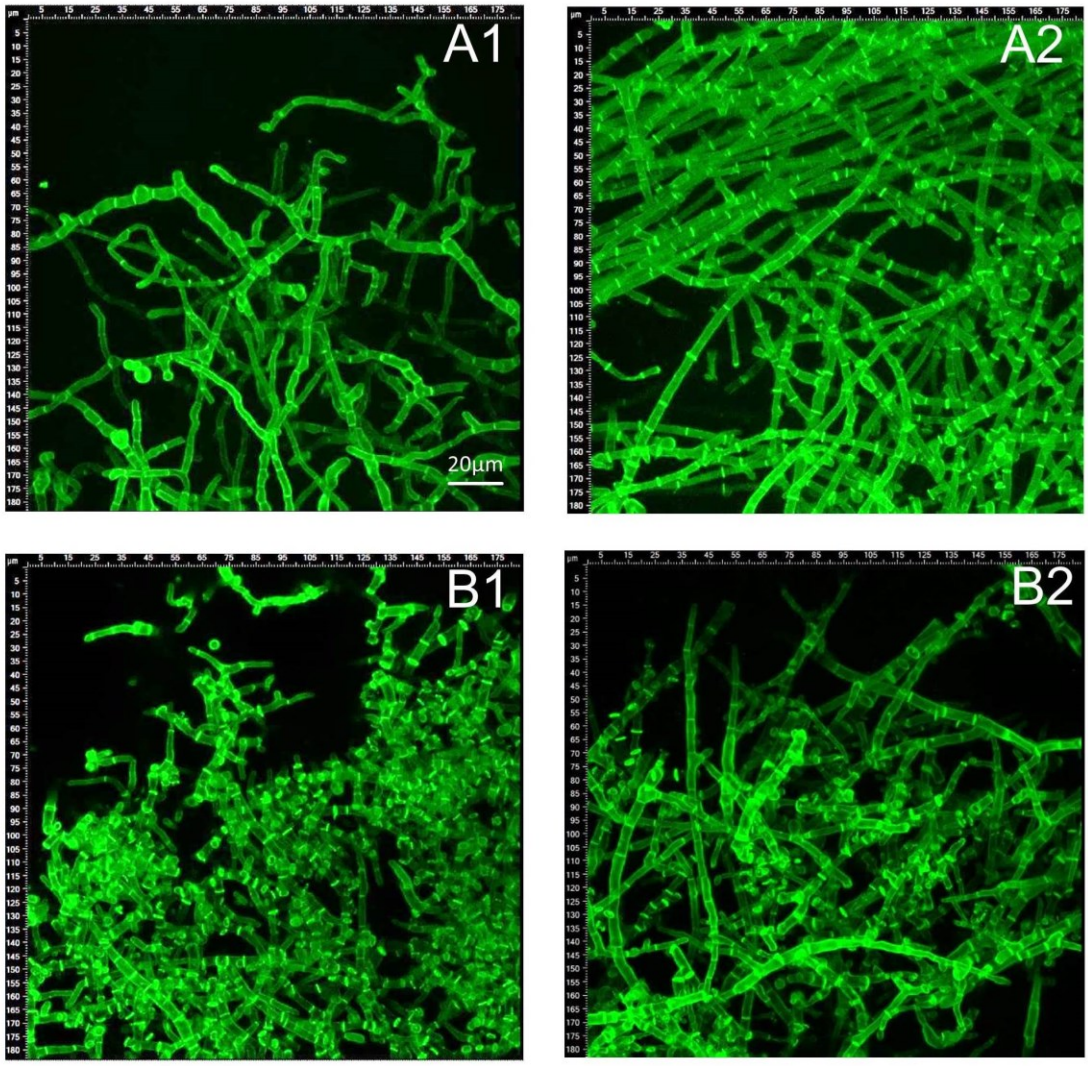


Figure 2 - CLSM images of lactose cultivations by *T. reesei* strains QM9414 (A1 and A2) and Δcre (B1 and B2)

Precultures were inoculated with 10^5 spores/mL (A1 and B1) or with a piece of overgrown agar (A2 and B2). All cultivations were incubated at 190 rpm.

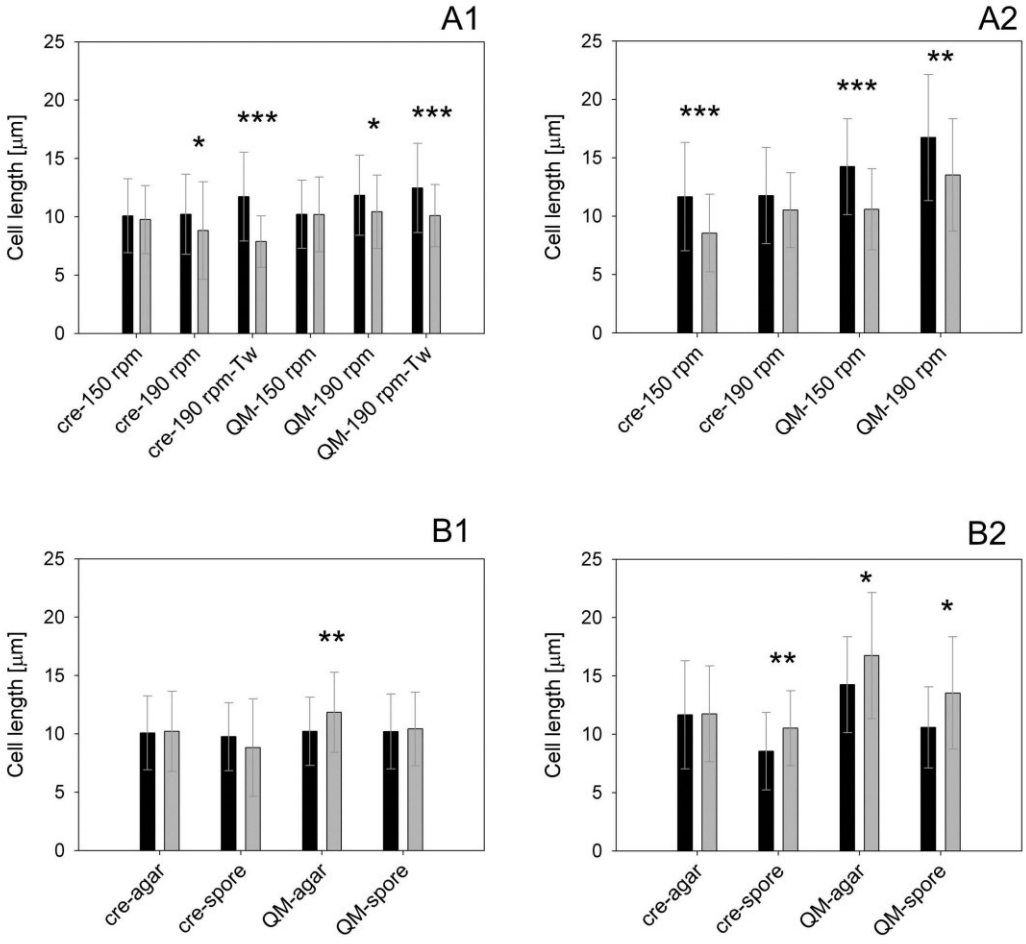


Figure 3 - The influence of the inoculation method (A1 and A2) and the agitation velocity (B1 and B2) on cell length

Cell length was analyzed in wheat straw (A1 and B1) and lactose (A2 and B2) cultivations. **A:** Precultures were inoculated with a piece of overgrown agar (black bars) or 10^5 spores/mL (grey bars). **B:** The agitation velocity was 150 rpm (black bars) or 190 rpm (grey bars) in an orbital incubator shaker. Cultivations were accomplished with *T. reesei* strain Δcre ("cre") or QM9414 ("QM"). Incubation was at 150 rpm or 190 rpm, as indicated. Addition of Tween80 is marked with "Tw". Data were taken from Table 1, where bars indicate the mean length and error bars show the

spread. Data sets with 1, 2 or 3 stars mark significance in a 95%, 99 % and 99.9 % confidence level, respectively.

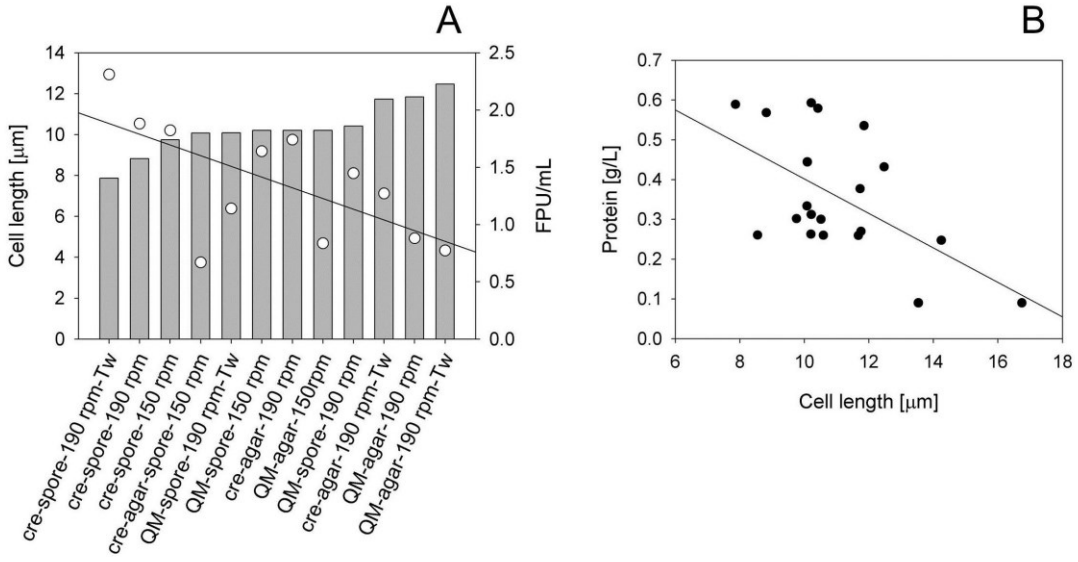


Figure 4 - Correlation between cell length and total cellulase activity in wheat straw cultivations (A) and total protein concentration in wheat straw and lactose cultivations (B)

A: Depicted are cell length (grey bars) and total cellulase activity (empty circles). Cultivations were accomplished with *T. reesei* strain Δcre ("cre") or QM9414 ("QM"). Inoculation was with a piece of overgrown agar ("agar") or 10^5 spores/mL ("spores"). Incubation was at 150 rpm and 190 rpm, as indicated. Addition of Tween80 is marked with "Tw". **B:** Depicted are cell length and the corresponding protein concentration in the supernatant of all experimental setups. Data are taken from Table 1.

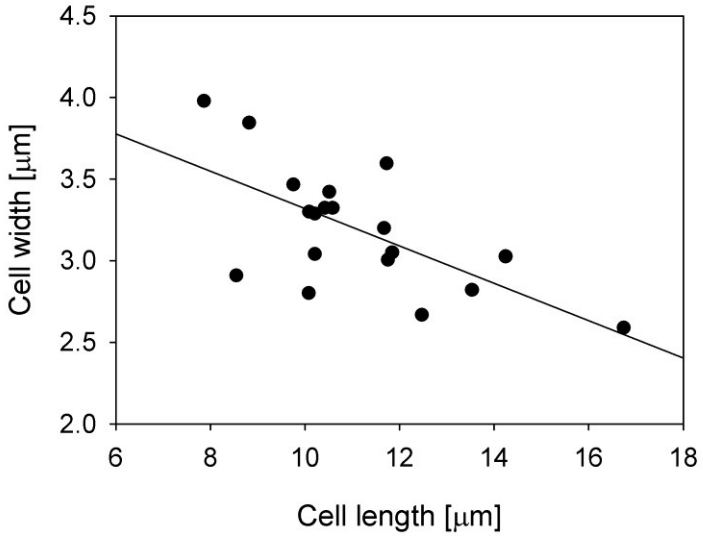


Figure 5 - Correlation between cell length and cell width

Figures include all data points as presented in Table 1.

Chapter 1

Supplementary information

Additional file 1: SI-1

Differences in macromorphology in cultivations on lactose and wheat straw

Additional file 2: SI-2

CLSM images of *T. reesei* strain Δcre after 2 and 8 days of lactose cultivation

Additional file 3: SI-3

CLSM image of fungal growth on wheat straw

Additional file 4 SI-4

CLSM images of *T. reesei* strain QM9414 after 8 and 14 days of lactose cultivation

Additional file 5 SI-5

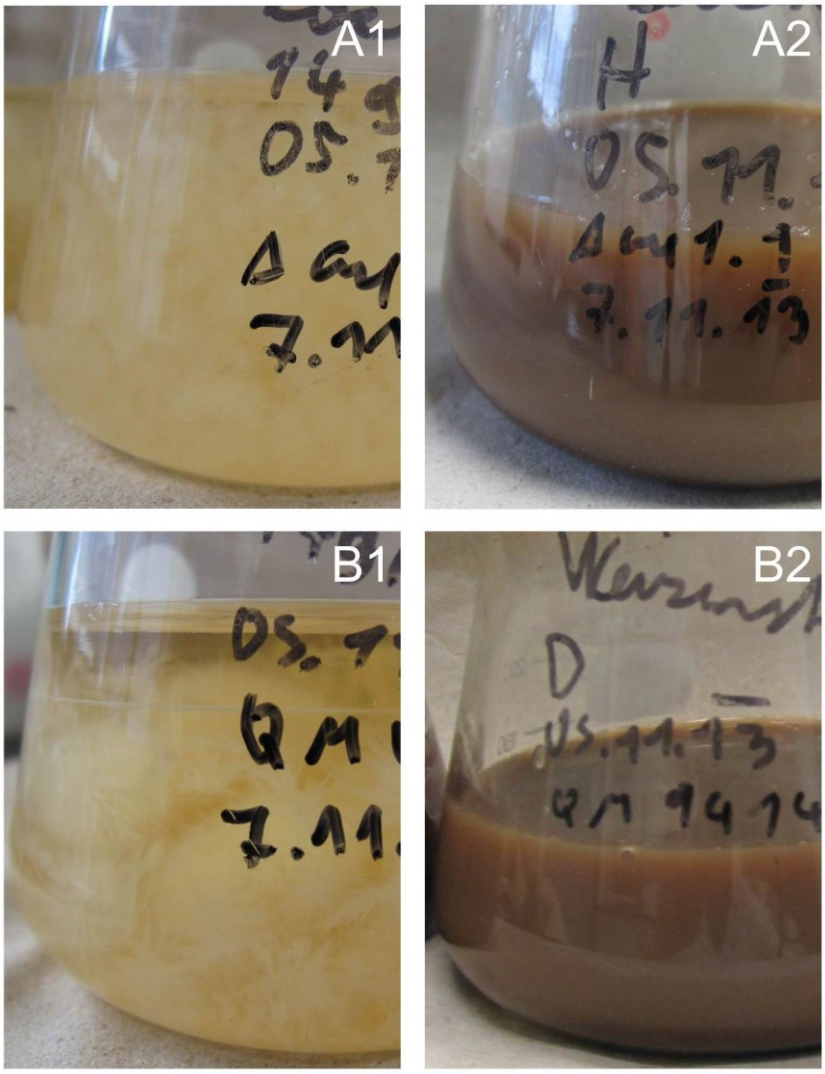
Protein and biomass production in cultivations by *T. reesei* strains Δcre and QM9414

Additional file 6 SI-6

The interface of the MATLAB program and an example of the resulting 3-dimensional hyphae skeleton

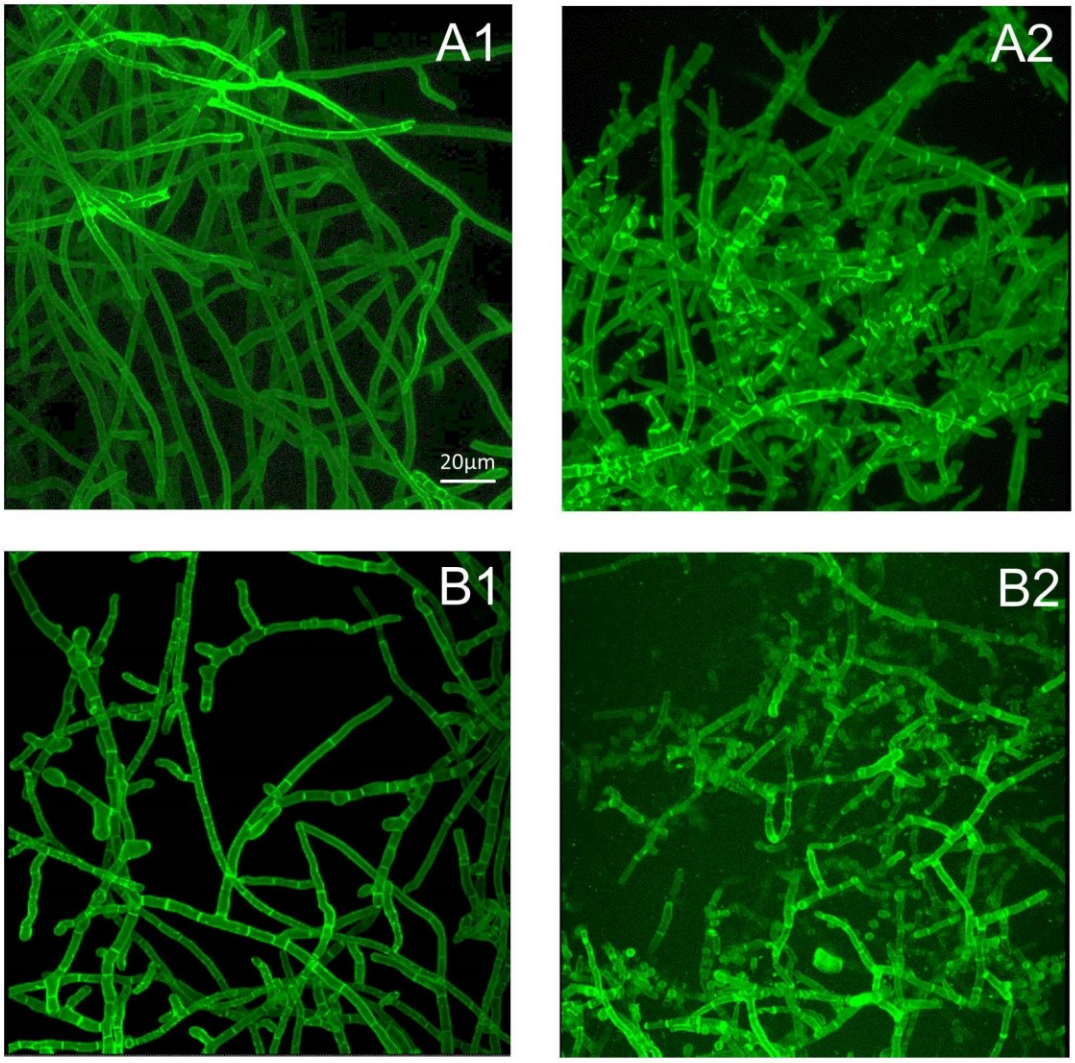
Additional file 7 SI-7

Cell length distribution



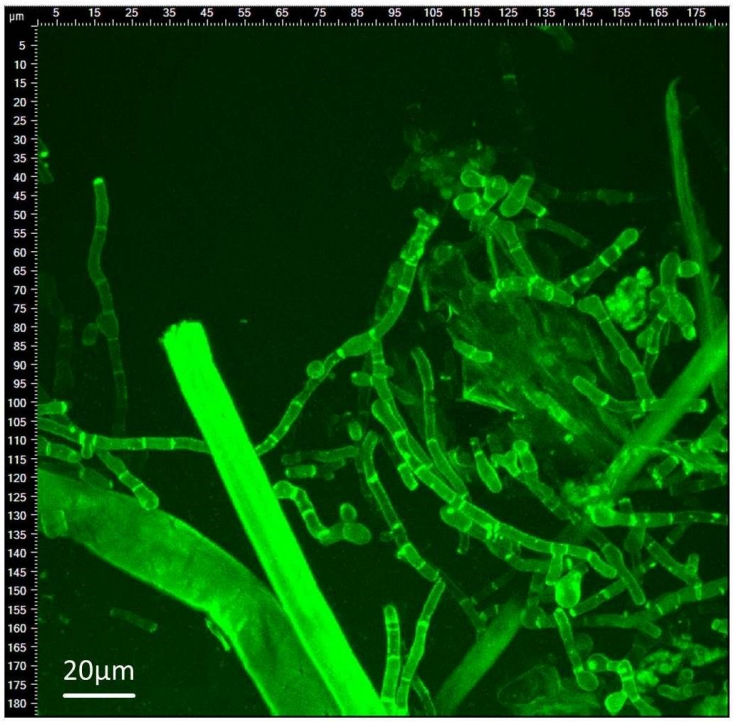
Additional file 1 - Differences in macromorphology in cultivations on lactose (A1 and B1) and wheat straw (A2 and B2)

Depicted are cultivations by *T. reesei* strains Δcre (A1 and A2) and QM9414 (B1 and B2), directly inoculated with 10^5 spores/mL and incubated at 190 rpm.



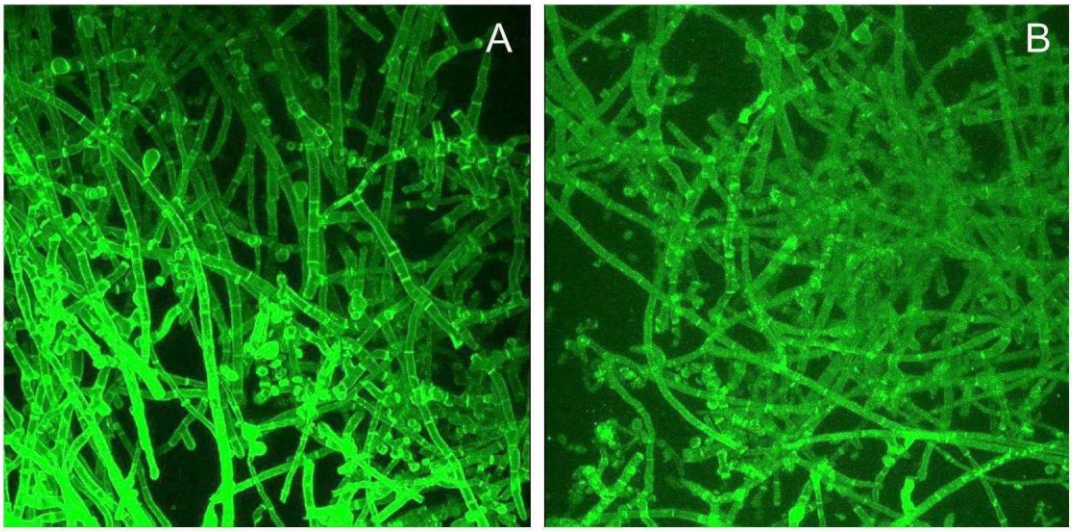
Additional file 2 - CLSM images of *T. reesei* strain Δcre after 2 and 8 days of lactose cultivation

Cultivations were directly inoculated with a piece of overgrown agar (panels A1 and A2) or 10^5 spores/mL (panels B1 and B2). Samples were taken after 2 days (panels A1 and B1) and 8 days (panels A2 and B2) of cultivation. Incubation was at 190 rpm.



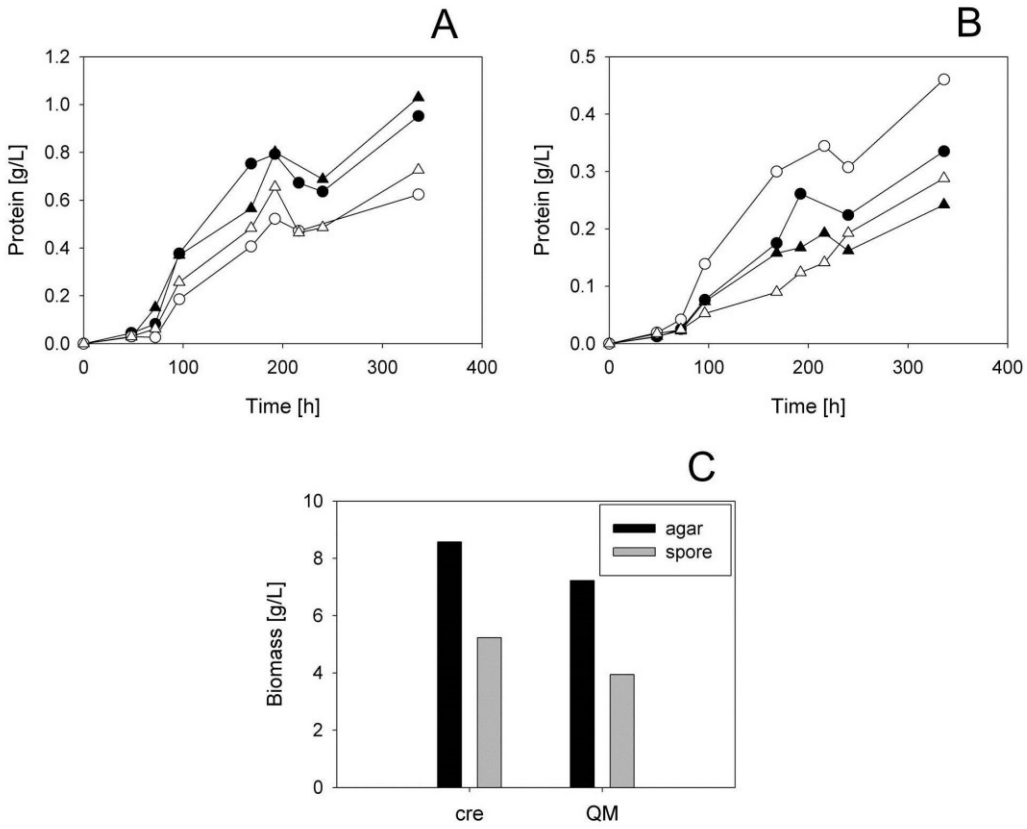
Additional file 3 - CLSM image of fungal growth on wheat straw

Cultivations on wheat straw by *T. reesei* strain QM9414. The culture was directly inoculated with a piece of overgrown agar and incubated at 190 rpm.



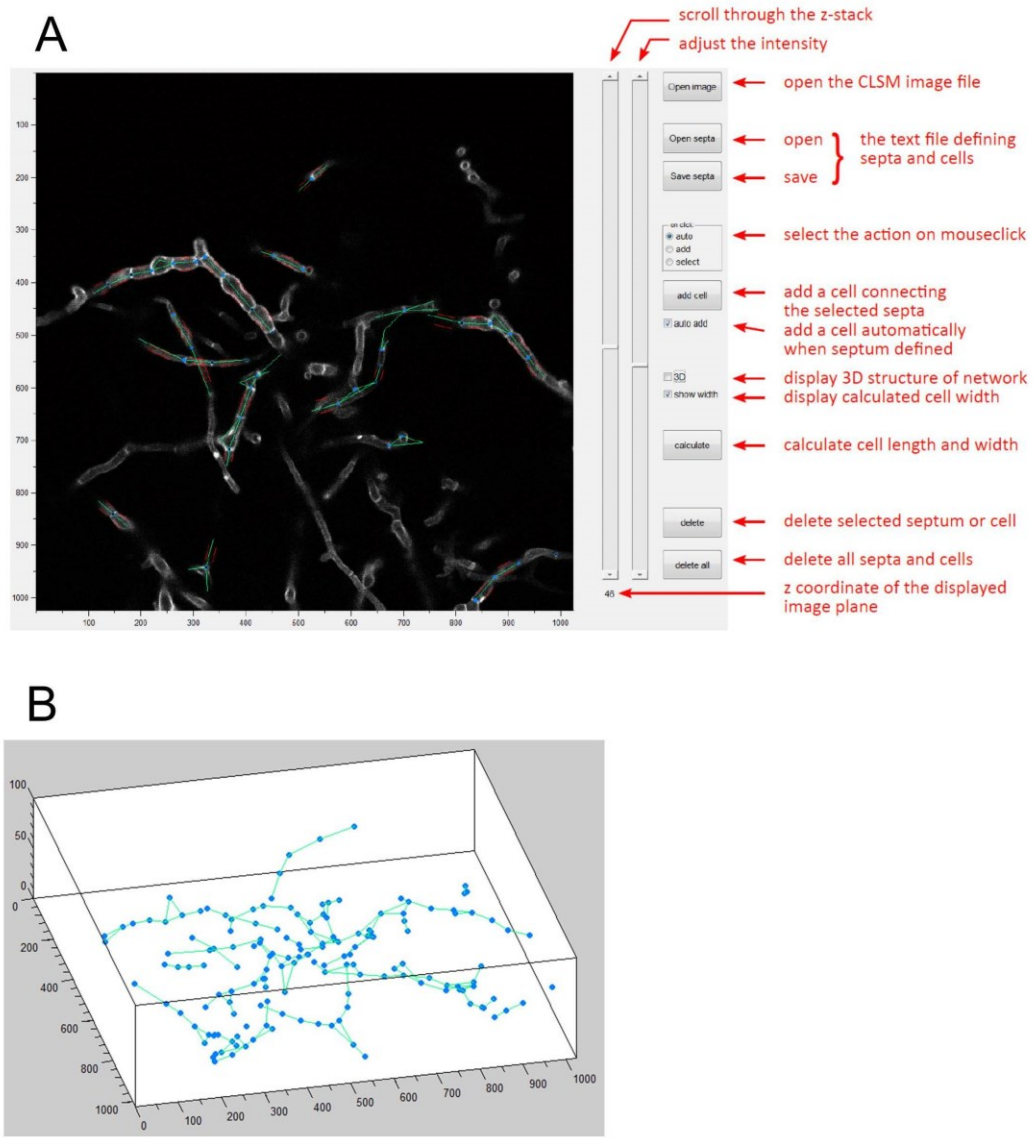
Additional file 4 - CLSM images of *T. reesei* strain QM9414 after 8 (A) and 14 (B) days of lactose cultivation

Cultivations were directly inoculated with a piece of overgrown agar and were incubated at 190 rpm.



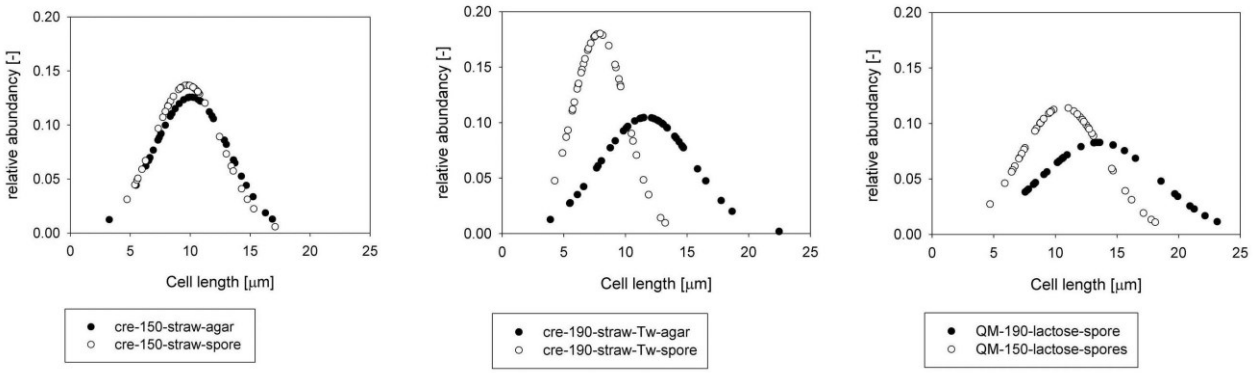
Additional file 5 - Protein (A and B) and biomass (C) production in cultivations by *T. reesei* strains Δcre and QM9414.

A and B: Depicted are the protein concentrations over time in wheat straw (A) and lactose (B) cultivations by *T. reesei* strains Δcre (circles) and QM9414 (triangles). Cultures were directly inoculated with 10^5 spores/mL (open symbols) or a piece of overgrown agar (close symbols). **C:** The final biomass (wet weight) concentration was measured in lactose cultivations after 200 h of incubation. Cultivations were directly inoculated with a piece of overgrown agar (black bars) or 10^5 spores/mL (grey bars). Incubation for all experiments was at 190 rpm.



Additional file 6 - The interface of the MATLAB program (A) and an example of the resulting 3-dimensional hyphae skeleton (B)

A: A screenshot of the MATLAB program for processing the CLSM images with a short description of the control elements. **B:** A 3-dimensional representation of the hyphae skeleton after the image processing with the MATLAB program.



Additional file 7 - Cell length distribution

Examples of the normal distribution of the cell lengths in wheat straw and lactose cultivations as indicated.

Chapter 2

Process intensification through microbial strain evolution: mixed glucose-xylose fermentation in wheat straw hydrolyzates by three generations of recombinant *Saccharomyces cerevisiae*

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Process intensification through microbial strain evolution: mixed glucose-xylose fermentation in wheat straw hydrolyzates by three generations of recombinant *Saccharomyces cerevisiae*

Vera Novy, Stefan Krahulec, Manfred Wegleiter, Gerdt Müller, Karin Longus, Mario Klimacek and Bernd Nidetzky*

Abstract

Background: Lignocellulose hydrolyzates present difficult substrates for ethanol production by the most commonly applied microorganism in the fermentation industries, *Saccharomyces cerevisiae*. High resistance towards inhibitors released during pretreatment and hydrolysis of the feedstock as well as efficient utilization of hexose and pentose sugars constitute major challenges in the development of *S. cerevisiae* strains for biomass-to-ethanol processes. Metabolic engineering and laboratory evolution are applied, alone and in combination, to adduce desired strain properties. However, physiological requirements for robust performance of *S. cerevisiae* in the conversion of lignocellulose hydrolyzates are not well understood. The herein presented *S. cerevisiae* strains IBB10A02 and IBB10B05 are descendants of strain BP10001, which was previously derived from the widely used strain CEN.PK 113-5D through introduction of a largely redox-neutral oxidoreductive xylose assimilation pathway. The IBB strains were obtained by a two-step laboratory evolution that selected for fast xylose fermentation in combination with anaerobic growth before (IBB10A02) and after adaption in repeated xylose fermentations (IBB10B05). Enzymatic hydrolyzates were prepared from up to 15% dry mass pretreated (steam explosion) wheat straw and contained glucose and xylose in a mass ratio of approximately 2.

Results: With all strains, yield coefficients based on total sugar consumed were high for ethanol (0.39 to 0.40 g/g) and notably low for fermentation by-products (glycerol: ≤0.10 g/g; xylitol: ≤0.08 g/g; acetate: 0.04 g/g). In contrast to the specific glucose utilization rate that was similar for all strains ($q_{Glucose} \approx 2.9$ g/g$_{cell\ dry\ weight\ (CDW)}$/h), the xylose consumption rate was enhanced by a factor of 11.5 (IBB10A02; $q_{Xylose} = 0.23$ g/g$_{CDW}$/h) and 17.5 (IBB10B05; $q_{Xylose} = 0.35$ g/g$_{CDW}$/h) as compared to the q_{Xylose} of the non-evolved strain BP10001. In xylose-supplemented (50 g/L) hydrolyzates prepared from 5% dry mass, strain IBB10B05 displayed a q_{Xylose} of 0.71 g/g$_{CDW}$/h and depleted xylose in 2 days with an ethanol yield of 0.30 g/g. Under the conditions used, IBB10B05 was also capable of slow anaerobic growth.

Conclusions: Laboratory evolution of strain BP10001 resulted in effectively enhanced q_{Xylose} at almost complete retention of the fermentation capabilities previously acquired by metabolic engineering. Strain IBB10B05 is a sturdy candidate for intensification of lignocellulose-to-bioethanol processes.

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Background

Second-generation biofuel production aims at biotechnological conversion of lignocellulosic biomass into liquid fuels, typically ethanol. Processes currently advancing to commercial scale production are facing two problems in particular. Firstly, for an efficient release of fermentable sugars from the insoluble feedstock, a technically complex and energy-intensive series of upstream processing steps are required [1-3]. Hence, mechanical and thermochemical pretreatment methods, alone or in combination, are applied to degrade and remove the lignin and to enhance the accessibility of the structural carbohydrates hemicellulose and cellulose for the subsequent enzymatic hydrolysis. During this pretreatment, however, secondary decomposition processes lead to formation of by-products, for example furans, phenolic compounds and organic acids, and many of those are inhibitory or even toxic to microorganisms applied to sugar fermentation [1-3]. It is widely accepted therefore that lignocellulose hydrolyzates constitute exceptionally difficult substrates for biotechnological conversions [1,2,4,5]. Since intermediate purification of the hydrolyzate is usually not a viable process option, a key requirement for efficient second-generation bioethanol production is a microbial strain that combines useful fermentation capabilities with high robustness to the overall conditions of the hydrolyzate [1,2,4,5]. *Saccharomyces cerevisiae* is a sturdy ethanol producer with long-standing history in the fermentation industries. Among the different candidate microorganisms considered, therefore, *S. cerevisiae* stands out as a highly promising choice for industrial scale applications [6].

Even though the composition of fermentable sugars in lignocellulose hydrolyzates varies strongly depending on the feedstock and the upstream processing technology applied, it is typical for most substrates to contain a significant amount of xylose next to the main glucose [1]. A major limitation of *S. cerevisiae* for lignocellulosic bioethanol development is the organism's natural inability to utilize xylose. Metabolic engineering has therefore been key in the development of xylose-fermenting strains of *S. cerevisiae* [2,5-8]. The applied strategies can be classified broadly according to whether xylose assimilation, which occurs through net isomerization of xylose into xylulose, was achieved via two-step oxidoreductive or direct isomerase-catalyzed transformation, as shown in Figure 1 [9-13]. Recombinant strains derived from either strategy displayed the expected broadening of substrate scope towards xylose. However, their specific xylose uptake rates and ethanol formation rates were still very low in comparison to the corresponding specific rates on glucose [5-7]. Moreover, xylose fermentation capabilities were usually severely deteriorated upon switching from synthetic substrate conditions to the harsher conditions of a lignocellulosic hydrolyzate [2,6,8,14]. A particular downside of strains harboring the oxidoreductive pathway is that a substantial amount of xylose is converted to xylitol and thus lost for ethanol production. Xylitol by-product formation is widely believed to have its origin in a mismatched coenzyme usage, NADP or NAD, during xylose reduction and xylitol oxidation (Figure 1) [11,15-19]. Coenzyme specificity engineering in xylose reductase (XR; NADPH → NADH) and xylitol dehydrogenase (XDH; $NAD^+ \rightarrow NADP^+$) was useful to render the two-step isomerization of xylose a more nearly redox-neutral process (Figure 1) [15-17,20]. However, aside from the intended change in coenzyme specificity, engineered enzymes must also fulfill the requirement of good activity under physiological boundary conditions *in vivo*. Due to their favorable kinetic properties that include high turnover number and apparent substrate and coenzyme affinities well aligned to intracellular metabolite concentrations in xylose-fermenting *S. cerevisiae*, some of the reported XR variants are especially promising for strain development. This is exemplified clearly by strain BP10001 from this laboratory that harbors an optimized NADH-preferring mutant of *Candida tenuis* XR. BP10001 shows a xylitol yield

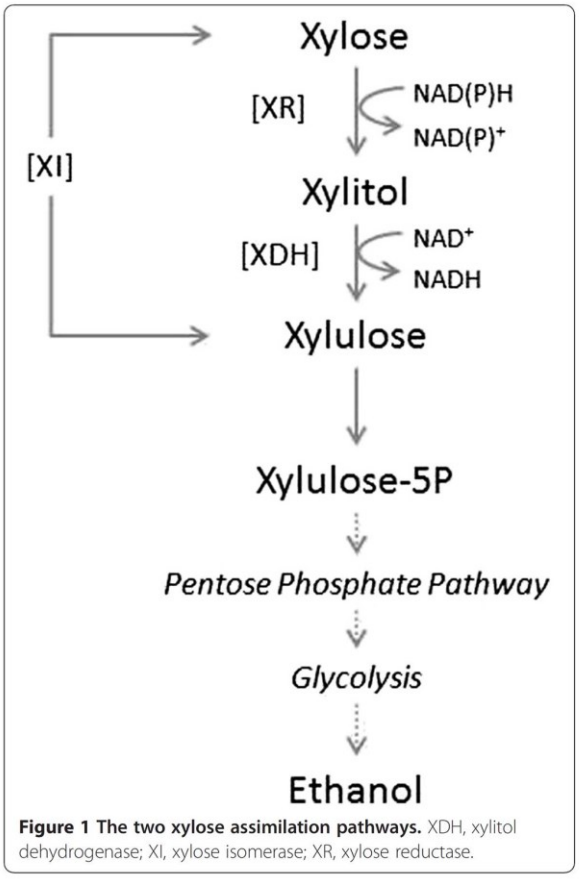

Figure 1 The two xylose assimilation pathways. XDH, xylitol dehydrogenase; XI, xylose isomerase; XR, xylose reductase.

($Y_{Xylitol}$) more than halved by comparison to $Y_{Xylitol}$ in the reference strain BP000 that expresses the NADPH-preferring wild-type XR [16,18-22]. Importantly, the lowering of $Y_{Xylitol}$ in BP10001 was not achieved in a trade-off against a decrease in xylose uptake rate (q_{Xylose}) [20,22]. However, q_{Xylose} of strain BP10001 was still almost two magnitude orders lower than the corresponding $q_{Glucose}$ and it was certainly insufficient for viable co-fermentation of glucose and xylose [20,22]. It must be emphasized that low q_{Xylose} is a common problem of xylose-fermenting strains of *S. cerevisiae*, regardless of the metabolic engineering strategy applied in their construction [6,7,13].

The specific substrate consumption rate is a kinetically complex rate parameter, which often eludes clear-cut dissection into one or more rate-determining transport or reaction steps, these in turn presenting distinct targets for further metabolic engineering. Laboratory evolution presents a long-known method for physiology optimization in microorganisms, and it has recently been adapted as a powerful complement of metabolic engineering to the development of high q_{Xylose} strains of *S. cerevisiae* [23-26]. Improvements in q_{Xylose} of up to one magnitude order were achieved using evolutionary engineering, and strains capable of co-utilization of glucose and xylose, and of cellobiose and xylose were obtained [25-28]. Interestingly, some strains also acquired the ability of slow anaerobic growth as a result of the evolution, presumably as a consequence of the enhanced ATP production rate at elevated q_{Xylose} [25,26]. Moreover, resistance to the overall environment of lignocellulose hydrolyzates or certain compounds present in it (for example furfural, acetic acid) could also be improved substantially by evolutionary engineering [23,29].

We have therefore applied laboratory evolution to strain BP10001 and used specific anaerobic growth rate (μ_{Xylose}) in combination with high q_{Xylose} to select strain IBB10A02 from several anaerobically growing yeast strains thus obtained. Further strain adaption through multiple rounds of batch xylose fermentations resulted in the identification of strain IBB10B05. The two evolved strains plus their parent strain BP10001 were compared in mixed glucose-xylose fermentation of undiluted and non-detoxified wheat straw hydrolyzate, which represents a notably challenging substrate. In Europe, wheat straw is considered to have the highest potential as biomass source for bioethanol production due to its abundance and low cost [30]. Even though wheat straw hydrolyzates have already been utilized as substrate for bioethanol production in the past, efficient xylose conversion often had complex process requirements, for example simultaneous saccharification and fermentation (SSF) [31,32] or fed-batch processing [33]. Therefore, further improvement in xylose conversion rates and higher ethanol yields at lower by-product formation must still be rendered possible [4-6,29,34]. To ensure optimal conditions for xylose fermentation and to keep technical requirements to a minimum level the process was run as separate hydrolysis and co-fermentation (SHCF) with the fermentation accomplished in simple batch cultures [33,34]. Results presented in this paper delineate marked gain in xylose fermentation efficiency and overall substrate tolerance due to evolutionary engineering, and strain IBB10B05 was identified as a promising candidate for direct glucose-xylose fermentation in unprocessed wheat straw hydrolyzate.

Results and discussion

Composition of the feedstock, and preparation of the sugar substrate for fermentation

Steam explosion has been described as an efficient and cost-effective method for the pretreatment of wheat straw [3,31-36]. Auxiliary chemical treatment is often applied to reinforce effectiveness of the steam explosion. We have applied here simple pretreatment based on steam explosion only. Table 1 shows the results of compositional analysis of the wheat straw after pretreatment. Dry matter content was about 20%, the water-insoluble portion thereof being roughly 68%. Pretreated feedstock composition was in agreement with literature data on wheat straw samples from different origin, but processed similarly [32,34,35].

Mixed sugar substrates for yeast fermentation were prepared by enzymatic saccharification of the pretreated wheat straw at a dry matter loading of 5% or 15% (by weight). Both fermentations were accomplished at the same boundary conditions, for example pH, temperature, agitation and starting OD_{600}. However, medium supplementation and sugar composition (glucose and xylose ratio) varied between the two hydrolyzates. We noticed that regardless of substrate and enzyme loadings applied to the saccharification, the resulting hydrolyzates always contained double the amount of glucose compared to xylose. Thus, 5% hydrolyzates contained glucose and xylose in concentrations of approximately 14 g/L and 7 g/L,

Table 1 Compositional analysis of the pretreated wheat straw

Component in dry matter	Percentage (%)
Carbohydrates	
Glucose	47.8
Xylose	21.3
Others	2.8
Non-carbohydrates	
Acid-soluble lignin	3.8
Acid-insoluble lignin	18.0
Ashes	1.5

respectively. To investigate xylose fermentation at elevated xylose/glucose (Xyl/Glc) ratio, like done in various studies from this and other groups in the past [32,34,35], we supplemented the 5% hydrolyzate to a final xylose concentration of approximately 50 g/L, resulting in a Xyl/Glc ratio of approximately 4. Mineral media, containing salts, vitamins and trace elements, was added to 5% hydrolyzates to ensure optimal fermentation conditions and maximal viability of the yeast [24,32,35]. However, in any larger scale process media additives such as salts or vitamins are economically and procedurally not feasible [2,14,37]. To address this problem, further fermentation studies were conducted under most simplified process and substrate conditions in the highly concentrated, non-detoxified 15% hydrolyzate with yeast extract as sole media supplement. As described previously, yeast extract serves as an excellent complex media additive for the fermentation of wheat straw hydrolyzates with high dry matter loadings [38]. Economically advantageous solutions, such as corn steep liquor or urea, in contrast, were described to be insufficient for wheat straw to bioethanol processes [38]. In this study, yeast extract served as a model for complex media supplementation, replacing the expensive mineral medium. However, addition of cheaper nutrient and nitrogen sources, for example grass juice, are future targets for process optimization.

Utilization of high substrate loadings is beneficial for lignocellulosic bioethanol production since the increase in sugar content (here: approximately 40 g/L and 20 g/L glucose and xylose, respectively in the 15% hydrolyzate) results in higher ethanol titers, which is important for facilitation of downstream processing. Throughout the manuscript, the sugar substrates used are identified as 15% hydrolyzate and 5% hydrolyzate$_X$, where subscript '$_X$' indicates externally added xylose.

Effect of *S. cerevisiae* strain evolution on xylose fermentation in basal medium

Strains IBB10A02 and IBB10B05 were obtained by laboratory evolution as described under Methods. The two strains were compared to their progenitor strain BP10001 by evaluating xylose (58 g/L) utilization in anaerobic shaken flask cultures. Time courses of fermentation product formation and biomass growth during xylose conversion were recorded for each strain, and the results are shown in Additional file 1. Compared to BP10001, the evolved strains displayed enhanced xylose fermentation capabilities in several respects. First of all, xylose consumption was markedly accelerated due to the combined effects of a distinct (≥2.5-fold) increase in q_{Xylose} and the establishment of anaerobic growth caused by the laboratory evolution (Table 2). q_{Xylose} values of about 1.0 g/g$_{CDW}$/h are among the highest reported for xylose-fermenting strains of *S. cerevisiae* [9,11,22,23,26,39].

Table 2 Comparison of μ_{max} and q_{Xylose} of strains BP10001, IBB10A02 and IBB10B05 obtained from xylose fermentation in YX medium

Parameter	BP10001	IBB10A02	IBB10B05
q_{Xylose} (g/g$_{CDW}$/h)	0.37 ± 0.05	0.98 ± 0.08	1.04 ± 0.06
μ_{max} (h^{-1})	n.d.	0.017 ± 0.001	0.021 ± 0.001

Data was obtained from two independent fermentations. n.d., not detectable.

Strain IBB10B05 grew faster and to a higher biomass concentration than strain IBB10A02 (Table 2, Additional file 1). In both strains, however, the specific growth rate on xylose (μ_{Xylose}) decreased strongly as xylose conversion progressed. Growth ceased completely at extended fermentation time (≥120 h), even though more than half of the initial xylose was still present and utilization of the sugar substrate continued further on. Considering that q_{Xylose} also decreased appreciably over the fermentation time course, shutdown of growth may reflect a drop of q_{Xylose} (and the ATP production rate associated with it) below a critical value. Additionally to its effects on key rate parameters of the fermentation (Table 2), we further analyzed the effect of laboratory evolution on the product distribution pattern of external metabolites produced from xylose. Data are summarized in Additional file 2. For all three yeast strains, the ethanol yield coefficient ($Y_{Ethanol/Xylose}$) was approximately 0.31 g/g. Yield coefficients for glycerol ($Y_{Glycerol/Xylose}$) were also similar for strains BP10001, IBB10A02 and IBB10B05 at approximately 0.04 g/g. Observed xylitol yields ($Y_{Xylitol/Xylose}$) were comparable for strain BP10001 and IBB10A02 (0.15 g/g) and increased 1.3-fold in fermentation utilizing strain IBB10B05 (0.19 g/g). The yield coefficients for strain BP10001 agree well with previously published results [22], indicating that the switch from mineral to yeast extract medium had no influence on product formation. Accordingly, mixed glucose-xylose conversion in spent sulfite liquor utilizing IBB10B05 was not affected by replacing mineral media with yeast extract [40]. Results of fermentation of xylose as the sole sugar substrate (Additional files 1 and 2, Table 2) strongly supported application of the two evolved yeast strains for mixed glucose-xylose conversion in wheat straw hydrolyzates. Conditions in the lignocellulose hydrolyzate are however noteworthy different from those of a pure glucose-xylose substrate (see, for example Palmqvist and Hahn-Hägerdal [4], Hahn-Hägerdal *et al.* [6], Casey *et al.* [41]), and the switch from defined to technological sugar substrates has proven to be difficult in the past [2,4,6].

Mixed glucose-xylose fermentation in xylose enriched 5% hydrolyzate$_X$: laboratory evolution results in markedly accelerated xylose utilization

A detailed time-course analysis for glucose-xylose fermentation in 5% hydrolyzate$_X$ was performed, comparing the

two evolved *S. cerevisiae* strains to the BP10001 reference. Results are summarized in Figure 2. All strains utilized glucose much faster than xylose. For clarity reasons, therefore, the respective 'glucose phase' was singled out and is shown in a separate graph (Additional file 3) depicting only the first phase (approximately 8 h) of the fermentation course. Determination of a specific glucose utilization rate ($q_{Glucose}$) for each strain was hampered due to rapid substrate depletion. However, time resolution of the shown data (Additional file 3) was sufficient to clarify that the three yeast strains consumed glucose at a comparable rate. Ethanol production ($Y_{Ethanol}$ = approximately 0.40 g/g) and glycerol formation ($Y_{Glycerol}$ = approximately 0.06 g/g) in the glucose phase were also similar for the different strains (Additional file 3). The specific growth rate ($\mu_{Glucose}$) for strain IBB10B05 approached a value expected for uninhibited *S. cerevisiae* growth during glucose fermentation, while in strains IBB10A02 and BP10001, the $\mu_{Glucose}$ values were notably decreased (Table 3). This provided the first evidence that strain IBB10B05 had gained superior resistivity to the conditions of the wheat straw hydrolyzate. Overall, IBB10B05 grew to a biomass concentration of approximately 2.6 g/L, which is significantly higher than reported for other yeast strains under comparable substrate conditions [34].

Evaluation of the second phase of the fermentation time courses, where xylose was utilized (Figure 2), revealed significant differences between the two evolved strains and their progenitor strain. The corresponding rate parameters and yield coefficients are summarized in Table 3. q_{Xylose} of IBB10A02 was enhanced 3.5-fold as compared to BP10001. IBB10B05 even surpassed IBB10A02 in terms of q_{Xylose}. The switch from pure xylose substrate to 5% hydrolyzate$_X$ caused a 2.5-fold decrease in q_{Xylose} for BP10001. In contrast, the evolved strains, particularly IBB10B05, showed a much less pronounced drop in q_{Xylose} (Table 3). It is noteworthy that both evolved strains consumed nearly all of the offered 50 g/L xylose within approximately 2 days. BP10001 by contrast showed much smaller xylose utilization (≤10 g/L) in the same time frame (Figure 2). Yield coefficients were similar for each strain, as shown in Table 3 where yield coefficients were calculated on the basis of total sugar, glucose and xylose consumed. Considering bias in the calculated yield coefficients due to unequal xylose utilization by BP10001 as compared to the evolved strains, we compiled a second set of yield coefficients (Additional file 4), which were determined from the xylose phase only, starting when the glucose was depleted fully. $Y_{Ethanol/Xylose}$ was about 0.31 ± 0.01 g/g in all strains and xylitol was the main by-product with $Y_{Xylitol/Xylose}$ in the range 0.18 to 0.23 g/g. Even though loss of xylose into xylitol formation was substantial with all three strains examined and therefore presents a clear target for strain optimization in the future, it is nevertheless worth noting that $Y_{Xylitol/Xylose}$ was not affected by change in substrate from pure xylose to 5% hydrolyzate$_X$. It was previously shown that enhanced burden on

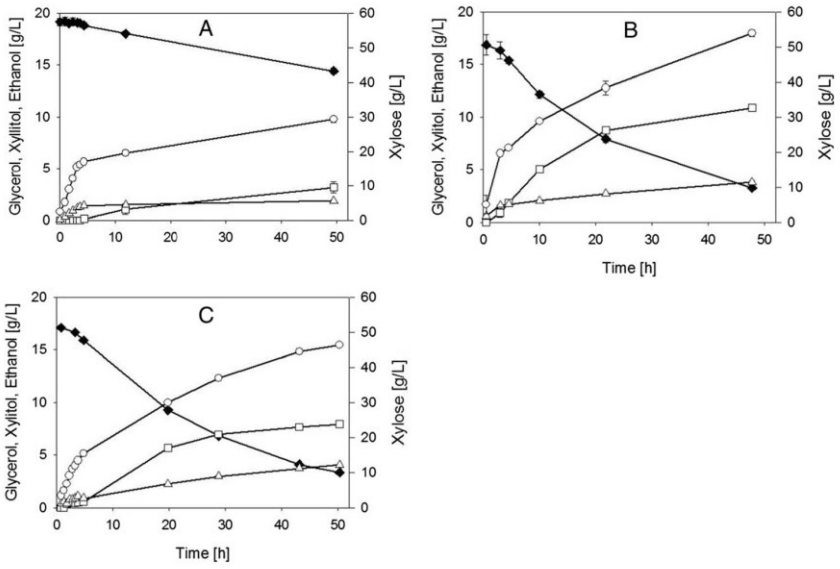

Figure 2 Time courses of mixed glucose-xylose fermentation in 5% hydrolyzate$_X$. Depicted are the first 50 h of fermentation using strains **(A)** BP10001, **(B)** IBB10A02 and **(C)** IBB10B05. Glucose (approximately 14 g/L) was depleted within the first 5 h and the 'glucose phase' is shown in Additional file 3. Data points are mean values of two independent fermentation experiments. Full diamonds, xylose; empty triangles, glycerol; empty squares, xylitol; empty circles, ethanol.

Table 3 Physiological parameters of strains BP10001, IBB10A02 and IBB10B05 obtained from mixed glucose-xylose fermentation in 5% hydrolyzate$_X$

Parameter	BP10001	IBB10A02	IBB10B05
$q_{Glucose}$ (g/g$_{CDW}$/h)	n.d.	n.d.	n.d.
q_{Xylose} (g/g$_{CDW}$/h)	0.15 ± 0.01	0.53 ± 0.05	0.71 ± 0.01
μ^a_{max}	0.09 ± 0.01	0.13 ± 0.02	0.43 ± 0.03
$Y_{Ethanol}$ (g/g)	0.35	0.31	0.30
($Y_{Ethanol/available\ sugars}$ (g/g))^b	(0.18)	(0.25)	(0.29)
$Y_{Glycerol}$ (g/g)	0.06	0.06	0.09
$Y_{Xylitol}$ (g/g)	0.15	0.22	0.17
$Y_{Acetate}$ (g/g)	0.04	0.06	0.05
Y_{BM} (g/g)	0.02	0.03	0.04
C-recovery (%)	98 ± 1	102 ± 1	96 ± 1

Data was obtained from two independent fermentations, mean errors of product coefficients were always below 11%. Fermentation time courses are shown in Figure 2. Fermentation in 5% hydrolyzate$_X$: glucose to xylose ratio of approximately 0.2.[a] Determined in the first 4.5 hours of fermentation;[b] $Y_{Ethanol/available\ sugars}$ = c (Ethanol produced in 100 h of fermentation)/c (Available glucose and xylose). Note that utilization of xylose was much lower in strain BP10001 than it was in the evolved strains. This affects the calculated ethanol yield coefficient based on total sugar (glucose and xylose) consumed. The ethanol yield coefficient was therefore also expressed based on total sugars available in the reaction, as shown in parenthesis. n.d., not detectable.

yeast physiology under conditions of a technological substrate could not only affect q_{Xylose}, but also result in increased xylitol yields at the expense of ethanol production [40]. In fact, this effect was also observed when substrate was altered from 5% hydrolyzate$_X$ to 15% hydrolyzate, as we will show hereinafter. Ethanol formation was almost doubled in the evolved strains compared to BP10001, whereby strain IBB10A02 accumulated up to 18 g/L ethanol within 2 days (Figure 2). The slightly smaller volumetric ethanol production by strain IBB10B05 compared to strain IBB10A02 is ascribed to experimental differences in the biomass concentration at the time of inoculation. Based on total sugar consumed, the obtained ethanol yield was about 70% of the theoretical value. Contrary to fermentations carried out with pure sugar substrate (Table 2), none of the three strains showed anaerobic growth on xylose in the 5% hydrolyzate$_X$. Although cell growth is an important feature of *S. cerevisiae* strains applied to lignocellulose-to-bioethanol processes [6,7,26], growth rates ($\mu_{Glucose}$ and μ_{Xylose}) are often excluded in literature [31-33,35], leading to the assumption that those yeast strains might have been growth impaired [34].

Mixed glucose-xylose fermentation in undiluted and non-detoxified 15% hydrolyzate: laboratory evolution confers a high degree of strain robustness

Results showing that laboratory evolution had caused enhancement of q_{Xylose} and consolidated anaerobic growth (on glucose) without compromising ethanol yield during mixed glucose-xylose fermentation of the 5% hydrolyzate$_X$ prompted us to take conversion experiments to another level of substrate complexity. Impairment of xylose fermentation in recombinant *S. cerevisiae* was previously described, when applying undiluted substrate at similar concentration as presented in this study [24,34,35], and this will also be confirmed for strain BP10001 hereinafter. Fermentation time courses recorded with the two evolved strains and strain BP10001 are shown in Figure 3. Specific rate and yield parameters calculated from the data are summarized in Table 4.

A recurring pattern in the fermentation time courses in Figure 3 was their division into two phases according to sugar substrate utilization. Glucose was consumed much faster than xylose. At the resolution of the experimental data with respect to time and concentration, sugar consumption appeared to have been largely sequential, glucose prior to xylose. It was shown in prior studies of BP10001 and also other xylose-fermenting strains of *S. cerevisiae* that low concentrations of glucose stimulate the uptake of xylose and only under these conditions a significant amount of true co-utilization of glucose and xylose becomes eventually possible [22,42]. However, at glucose concentrations at or higher than 5 g/L, xylose consumption is inhibited [22,42]. The evolved strains IBB10A02 and IBB10B05 do in fact show a small amount of glucose-xylose co-utilization at the end of their respective glucose phase (Figure 3). Strain BP10001 utilizes xylose at a much slower rate by comparison, thus resulting in a completely sequential fermentation pattern.

Recently *S. cerevisiae* harboring xylose isomerase (Figure 1) was evolved to a q_{Xylose} exceeding that of IBB10B05 in pure xylose substrate by still a factor of about 2 [39]. Glucose-xylose fermentation by the resulting yeast strain occurred at the transition between sequential and simultaneous utilization of hexose and pentose substrates, indicating that true co-fermentation may become possible at sufficiently high q_{Xylose}. However, fermentation of lignocellulose hydrolyzates was not examined, and evidence from this study suggests that q_{Xylose} is more strongly affected by substrate conditions than $q_{Glucose}$. Moreover, a number of publications on mixed glucose-xylose fermentation in lignocellulose hydrolyzates by recombinant *S. cerevisiae*, typically strains constructed using the XR/XDH pathway, agree with our findings of predominantly sequential sugar substrate utilization [33-35].

Even though fermentation of glucose was fast in each case (Figure 3), the three yeast strains differed in respect to $q_{Glucose}$ and $\mu_{Glucose}$, thus resulting in distinctly different efficiencies of glucose conversion. Strain IBB10B05 showed the highest $\mu_{Glucose}$ and the fastest glucose utilization (Figure 3, Table 4). $q_{Glucose}$ of strain IBB10A02 was surprisingly low. Analysis of the xylose phase from

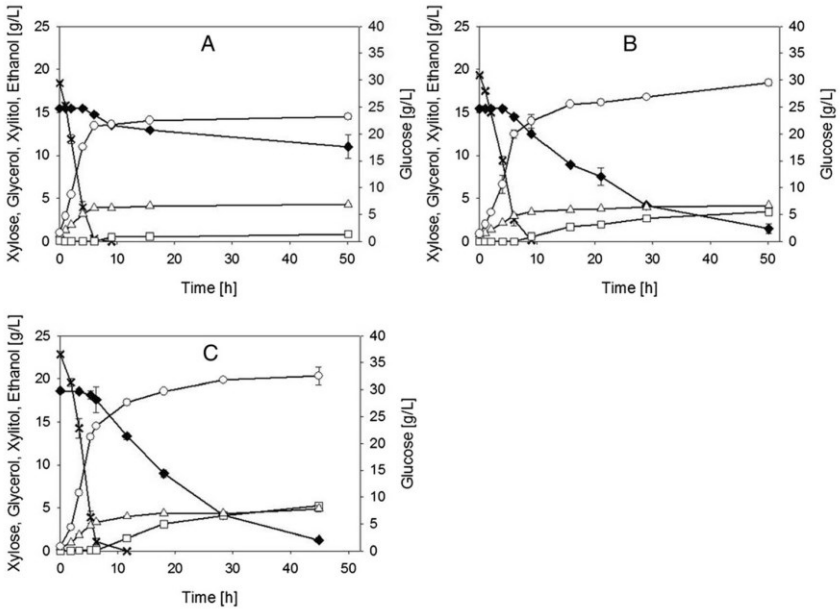

Figure 3 Time courses of mixed glucose-xylose fermentation in 15% hydrolyzate. Depicted are the first 50 h of fermentation utilizing strains **(A)** BP10001, **(B)** IBB10A02 and **(C)** IBB10B05. Data points are mean values of two independent fermentation experiments. Crosses, glucose; full diamonds, xylose; empty triangles, glycerol; empty squares, xylitol; empty circles, ethanol.

the 15% hydrolyzate conversion time course revealed dramatic effects of laboratory evolution on the yeast strains' xylose fermentation capabilities. While the progenitor strain BP10001 was utterly inefficient in utilizing xylose, the two evolved strains converted (nearly) all of the xylose present in 15% hydrolyzate within about two days. Expressed in q_{Xylose}, laboratory evolution brought about 11.5-fold (IBB10A02) and 17.5-fold (IBB10B05) enhancement of xylose utilization in 15% hydrolyzate as compared to BP10001. These improvement factors are of remarkable magnitude, and they therefore underscore the huge potential of evolutionary yeast strain engineering for biofuel process development.

Table 4 Physiological parameters of strains BP10001, IBB10A02 and IBB10B05 obtained from mixed glucose-xylose fermentation in 15% hydrolyzate

Parameter	BP10001	IBB10A02	IBB10B05
$q_{Glucose}$ ($g/g_{CDW}/h$)[a1]	2.81 ± 0.04	1.71 ± 0.05	2.90 ± 0.22
q_{Xylose} ($g/g_{CDW}/h$)	0.02 ± 0.01	0.23 ± 0.01	0.35 ± 0.02
μ_{max} [a2]	0.10 ± 0.01	0.10 ± 0.01	0.19 ± 0.01
$Y_{Ethanol}$ (g/g)	0.40	0.39	0.39
($Y_{Ethanol/available\ sugars}$ (g/g))[b]	(0.13)	(0.38)	(0.38)
$Y_{Glycerol}$ (g/g)	0.10	0.08	0.08
$Y_{Xylitol}$ (g/g)	0.04	0.08	0.08
$Y_{Acetate}$ (g/g)	0.04	0.04	0.04
Y_{BM} (g/g)	0.05	0.04	0.06
C-recovery (%)	103 ± 1	101 ± 1	104 ± 1

Data was obtained from two independent fermentations, mean errors of product coefficients were always below 14%. Fermentation time courses are shown in Figure 3. Fermentation in 15% hydrolyzate: glucose to xylose ratio of approximately 2.[a] Determined in the first [a1] 3.5 h and [a2] 6 hours of fermentation;[b] $Y_{Ethanol/available\ sugars}$ = c (Ethanol produced in 50 h of fermentation)/c (Available glucose and xylose).

Yield coefficients for mixed glucose-xylose fermentation in 15% hydrolyzate indicate good ethanol production ($Y_{Ethanol}$ approximately 0.40 g/g total sugars). Xylitol and glycerol were major by-products. A compilation of yield coefficients derived from the xylose phase is provided in Additional file 4. Compared to fermentations conducted in YX and 5% hydrolyzate$_X$ media, xylitol yields ($Y_{Xylitol/Xylose}$) in fermentations of the 15% hydrolyzate were notably elevated (up to 0.30 g/g; Additional files 2 and 4). The reason for high xylitol formation in 15% hydrolyzate was not further pursued.

Although direct comparison is difficult due to different feedstock applied, we noticed that $Y_{Ethanol}$ (approximately 0.40 g/g total sugars) and the final ethanol titer (approximately 20 g/L) for 15% hydrolyzate conversion by the evolved strains IBB10A02 and IBB10B05 (Table 4, Figure 3) were superior to the same fermentation parameters reported from other studies, where $Y_{Ethanol}$ did not exceed values of typically 0.37 g/g total sugars when undiluted substrate was applied, and final ethanol titers were below 10 g/L [34,35]. Higher ethanol yields as well as enhanced final ethanol titers were only achieved when

glucose was fed continuously to the fermentation mixture, by running the process in SSF [32,43] or separate hydrolysis and fermentation (SHF) run in fed-batch mode [33,34]. Consistently, we have shown that q_{Xylose} in mixed glucose-xylose fermentations by strain BP10001 is accelerated substantially by feeding low levels of glucose [22]. However, requirement for controlling the glucose feed tightly adds complexity of the process operation. Yeast strains producing high amounts of ethanol from xylose in simple batch fermentations are therefore of considerable interest for application in large-scale bioprocessing.

Strain evolution as tool for process intensification: comparison of yeast strain performance under different substrate conditions

Evidence from different studies, including the work presented herein, strongly supports the suggestion that evolutionary engineering of *S. cerevisiae* constitutes a powerful approach to achieve significant process intensification for xylose-to-ethanol fermentation [23-26,34,39]. Improvements in specific rate parameters and yield coefficients were shown to translate directly into pronounced enhancement of the final ethanol concentration, the process productivity, or both. Table 5 lists xylose-fermenting yeast strains generated by laboratory evolution and compares each strain to its corresponding progenitor. Consequences of evolutionary engineering were assessed in pure sugar substrate fermentations [23,26,39], but also in lignocellulose hydrolyzate conversions [24,34]. Results show that in a wide range of media and cultivation conditions using pure sugar substrates, evolution caused effective enhancement in q_{Xylose} (up to 8-fold) and conferred, or resulted in stabilization of, anaerobic growth on xylose (Table 5). The degree of xylose utilization and thus, the end concentration of ethanol were also increased by up to 4-fold. Even though the different studies are difficult to compare due to large variations in the experimental settings used, it is nonetheless clear that strain IBB10B05 features a notable

Table 5 Laboratory evolution of xylose-fermenting strains of *S. cerevisiae* as tool for process intensification: comparison of key process parameters reported for the progenitor strain and the evolved strain, respectively

Progenitor strain - evolved strain *Genetic background*[a]	Fermentation condition[b]	c_{Sugar} (g/L)[c]	q_{Xylose} ($g/g_{CDW}/h$)	$c_{Ethanol}$ (g/L)[d]	$Y_{Ethanol}$ ($g/g_{total\ sugars}$)	μ_{max} (h^{-1})[e]	Source
TMB3001 -	MM	Glc: 50	0.08*	22*	0.38*	0.44^{Glc}	
TMB3001C1	AN	Xyl: 50	0.31*	28*	0.40*	0.44^{Glc}	[26]
XR/XDH/XK	LCD		(3.9–fold)	(1.3–fold)	(1.1–fold)	(–)	
H131-$A3^{SB-2}$ -	YE	Xyl: 40	0.26	4*	0.42	0.06^{Xyl}	
H131-$A3^{CS}$	AN		0.94	15*	0.43	0.12^{Xyl}	[39]
XI/ PPP/ T	LCD		(3.6–fold)	(3.8–fold)	(–)	(2–fold)	
HDY.GUF5 -	YE, Pep	Glc: 36	0.13	18*	0.23	n.a.	
GS1.11-26	Semi-AN	Xyl: 37	1.10	34*	0.46	n.a.	[23]
XI/PPP	HCD		(8.5–fold)	(1.9–fold)	(1.8–fold)	(–)	
BP10001 -	YE	Xyl: 50	0.37	0.9	0.30	-	
IBB10B05	AN		1.04	2.8	0.31	0.02^{Xyl}	This study
XR/XDH/XK	LCD		(2.8–fold)	(3.1–fold)	(–)	(–)	
TMB3400 -	Wheat straw hydrolyzate, YE, salts, pH 5	Glc: 7.6	0.20*	5.5	0.20	-	
KE6-13i	AN	Xyl: 38	0.04*	6	0.27	-	[34]
XR/XDH/XK	HCD		(-)	(1.1–fold)	(1.4–fold)	(–)	
TMB3400 -	Spruce hydrolyzate, MM, pH 5		n.a.	7.8*	0.40	0.07^{Glc}	
KE1-17	AN	Glc: 18	n.a.	7.9*	0.43	0.08^{Glc}	[24]
XR/XDH/XK	LCD	Xyl: 9	(–)	(–)	(1.1–fold)	(1.14–fold)	
BP10001 -	Wheat straw hydrolyzate, YE, pH 6.5		0.02	4	0.40	0.10^{Glc}	
IBB10B05	AN	Glc: 32	0.35	21	0.39	0.19^{Glc}	This study
XR/XDH/XK	HCD	Xyl: 16	(17.5–fold)	(5.3–fold)	(–)	(1.9–fold)	

[a]Strain background: PPP, overexpression of genes from the pentose phosphate pathway; T, overexpression of the HXT7 transporter; XDH, xylitol dehydrogenase; XI, xylose isomerase; XK, xylulose kinase; XR, xylose reductase. [b]AN, anaerobic; HCD, high cell density (start OD_{600} of fermentation ≥1); LCD, low cell density (start OD_{600} of fermentation ≤0.5); MM, mineral medium; Pep, peptone; YE, yeast extract. [c]Initial sugar concentration of the substrate. [d]Ethanol produced within the first 50 h of fermentation or earlier, when sugars were depleted before. [e]Maximal growth rate on glucose (Glc) or xylose (Xyl). n.a., not analyzed; in parenthesis, improvement calculated from the evolved strain as compared to the progenitor strain; *Data are derived from the time courses given in the respective publications.

overall improvement in xylose fermentation capability. Effectiveness of the evolutionary procedure in IBB10B05 was remarkable in particular, considering that development of the progenitor strain BP10001 had involved only a minimum amount of metabolic engineering of the parent strain *S. cerevisiae* CEN.PK 113-5D. Interestingly, change of the sugar substrate to an unprocessed concentrated wheat straw hydrolyzate resulted in substantial (≥2-fold) reinforcement of the process intensification effect of the strain evolution. This is reflected in very pronounced enhancement of q_{Xylose}, the completeness of xylose consumption, and the final ethanol titer achievable with strain IBB10B05 as compared to strain BP10001. This result serves to emphasize the high robustness acquired by strain IBB10B05 during evolutionary engineering, despite the fact that increased resistance to conditions of the hydrolyzate was not selected for. Remarkably enough, yeast strain evolutions reported from other laboratories to specifically address tolerance against biomass-derived inhibitors did not achieve comparable improvements of strain performance during fermentation of lignocellulose hydrolyzates [24,34]. The lignocellulosic ethanol concentration of 21 g/L reached with strain IBB10B05 therefore surpassed comparative values in Table 5 by 3-fold or more.

Conclusions

The q_{Xylose} is a complex physiological parameter of key technological importance in *S. cerevisiae* fermentations of lignocellulose hydrolyzates. Laboratory evolution of strain BP10001 to generate strains IBB10A02 and IBB10B05 resulted in effectively (up to 17.5-fold) enhanced q_{Xylose} at complete perpetuation of the fermentation capabilities ($Y_{Ethanol}$; $q_{Glucose}$) previously acquired by metabolic engineering. Strain IBB10B05 was identified as a particularly robust candidate for intensification of lignocellulose-to-bioethanol production processes.

Methods

Chemicals and media used

Unless mentioned otherwise, all chemicals were from Carl Roth + Co KG (Karlsruhe, Germany). Defined mineral (M-) medium was prepared as described elsewhere [15], except that riboflavin and folic acid were not added. For use of M-medium under anaerobic conditions, ergosterol (10 mg/L), Tween 80 (0.42 g/L) and 250 μL/L Antifoam 204 (all from Sigma-Aldrich, St Louis, MO, USA) were additionally supplied. YPD medium contained yeast extract (10 g/L), peptone (from casein, 20 g/L) and glucose (20 g/L). YX medium contained yeast extract (10 g/L) and xylose (58 g/L). Medium for anaerobic agar plate cultivation contained yeast extract (8 g/L), peptone (from casein, 10 g/L), xylose (20 g/L), agar (13 g/L), thioglycolate (500 mg/L), L-cysteine (500 mg/L) and resazurin (1 mg/L). All media were brought to pH 6.5, and the pH was verified after sterilization.

Laboratory evolution of strain BP10001 and isolation of strains IBB10A02 and IBB10B05

Strain BP10001 was previously constructed from *S. cerevisiae* CEN.PK 113-5D through genomic integration of genes encoding a doubly mutated (Lys^{274}-to-Arg; Asn^{276}-to-Asp) variant of XR from *C. tenuis* and the wild-type XDH from *Galactocandida mastotermitis*. Another gene copy of the endogenous xylulose kinase 1 was also integrated. Each gene was expressed under control of the constitutive TDH promoter and the CYC1 terminator. Laboratory evolution was carried out with strain BP10001 in two steps. Because evolutionary engineering of BP10001 will be described in a separate paper, only a brief summary is given here. Firstly, strain BP10001 was incubated in 15 mL glass tubes (Pyrex® Brand 9825) containing 10 mL M-medium supplemented with 50 g/L xylose (XM). Each tube was inoculated to a cell density of 0.04 g_{CDW}/L and incubation was at 150 rpm in a CERTOMAT BS-1 incubator shaker (Sartorius AG, Göttingen, Germany) at 30°C for 91 days. Afterwards, 400 μL of cell suspension were plated on agar and incubated in an anaerobic jar at 30°C for 15 days. Single colonies were picked, transferred to new agar plates and further incubated for 5 days. Fast growing colonies were selected for cultivation in tubes as described above. The strain showing the highest μ_{max} (strain IBB10A02), determined as the increase in optical density at 600 nm (OD_{600}) over time, was used for further evolutionary engineering by repetitive batches. Hence, strain IBB10A02 was grown (start OD_{600} approximately 0.05) under anaerobic conditions in sealed flasks containing XM-medium. At mid-exponential phase (OD_{600} approximately 1), cells were transferred to a new batch (OD_{600} approximately 0.05) containing fresh XM-medium. Cells were again cultivated until the mid-exponential phase was reached. This procedure was repeated until the observed μ_{max} was approximately doubled. Positive strains were isolated under anaerobic conditions and tested with respect to μ_{max} and $Y_{Xylitol}$ and the best performing strain was termed IBB10B05.

Preparation of the lignocellulosic feedstock

Austrian wheat straw was utilized. The wheat straw was air-dried to a water content of approximately 10% (w/w) and the fibers were chaffed in a shredder (GE 365; Viking, Tyrol, Austria) to reduce the fiber length to an average of 3 to 4 cm. Further, the wheat straw was treated by steam explosion at 200°C, 15 bar for 10 min with a water to wheat straw ratio of 3. After cooling the wheat straw was stored at −20°C in plastic bags. Dry mass (DM) and water-insoluble content (WIS) were

analyzed in triplicates. For DM determination, a moisture analyzer operated at 105°C (MA 50; Sartorius AG) was used. For WIS determination, 2 g of the wheat straw was washed with 50 mL of 50°C warm water, dried at 105°C for 24 h and weighed. Additionally, the content of structural carbohydrates, lignin and ash in the wheat straw was analyzed in double determination, following the protocol of the National Renewable Energy Laboratory (NREL) [44]. The resulting compositional analysis is depicted in Table 1. Besides the main sugars glucose and xylose, only small amounts of mannose (<1.9% DM wheat straw) and arabinose (<0.9% DM wheat straw) could be detected, and they are summarized as 'others' in Table 1.

Enzymatic hydrolysis

Enzymes for wheat straw hydrolysis were produced using the *Trichoderma reesei* strain SVG17 as described previously [45]. Briefly, the fungus was cultivated in a BIOSTAT C4 bioreactor (Sartorius AG) with 5 L working volume. Pretreated wheat straw (3% (w/v)) was the sole carbon source. Fermentations were run for 7 to 9 days (30°C, pH 4.5, 20% dissolved oxygen), until no further increase in cellulase activity was detected. Cellulase activity was measured with the filter paper unit (FPU) assay as recommended by the International Union of Pure and Applied Chemistry (IUPAC) [46]. The enzyme solution was harvested by centrifugation (4,420 *g*, 4°C, 20 min, Sorvall RC-5B; DuPont Instruments, Wilmington, DE, USA) and the supernatant filtered sterile (Whatman Klari-Flex System; GE Healthcare, Little Chalfont, UK). Hydrolyzates were freshly prepared shortly before fermentation from one batch of pretreated wheat straw. The substrate loading was 5% and 15% DM wheat straw and the enzyme loading was 25 FPU/g DM. Reaction was performed in 10 mM sodium acetate buffer (pH 4.8) in 500 mL shaken flasks with ground in stoppers filled with 200 mL wheat straw suspension. The wheat straw suspension was autoclaved and the enzyme solution sterile filtrated. Incubation was at 50°C and 200 rpm in an incubator shaker (CERTOMAT BS-1) for 48 h. Afterwards, the hydrolyzate was heated to 100°C for 15 min and remaining solids were removed by centrifugation (4,420 *g*, 4°C, 10 min, Sorvall RC-5B). The pH of the hydrolyzate was set to 6.5 with 1 M NaOH solution. The sugar content of the hydrolyzates was analyzed by HPLC as described below. We noted variation in the composition of the 15% hydrolyzates prepared in different hydrolysis runs (N >10). Glucose and xylose were present at 42.8 ± 3.9 g/L and 21.1 ± 3.1 g/L, respectively. Acetic acid concentration was 3.6 ± 0.5 g/L. Mannose (<0.7 g/L) and galactose (<0.2 g/L) were present in small amounts. Cellobiose showed the highest variation in the range 1 to 5 g/L. Activity of β-glucosidase, which is the enzyme hydrolyzing cellobiose into glucose, may have been limiting in some of the cellulase preparations applied to hydrolysis. Reasons for variation in sugar content of different hydrolyzates are not completely clear at this time, and their examination was left for consideration in the future. However, each yeast strain was used in multiple fermentation experiments (N ≥3) and the reported parameters were not affected significantly by the relevant variations in hydrolyzate composition.

Shaken bottle fermentations

Reactions were performed anaerobically at 30°C. About 80% of the total volume was wheat straw hydrolyzate and the remainder volume was composed of media supplementation (10%) and inoculum (10%). In fermentations of 5% hydrolyzate, M-medium and xylose (58 g/L) were added (5% hydrolyzate$_X$). The 15% hydrolyzate fermentations were supplemented with yeast extract (10 g/L). Starting OD_{600} in fermentations of the hydrolyzates was 5. Additionally, fermentations were conducted in YX media, with a starting OD_{600} of 0.5. Seed and starter cultures were prepared in M-media with additional glucose (20 g/L) for fermentations supplemented with mineral media. All others were prepared in YPD media. Yeast strains were stored at −70°C in glycerol stocks and initially plated on YPD agar. Incubation was at 30°C for 48 h. Afterwards, cells were transferred to 500 mL shaken flasks filled with 50 mL of the respective media and incubated at 30°C overnight. Subsequently, cells were transferred to 300 mL of fresh media in 1,000 mL shaken flasks. Starting OD_{600} was 0.05 and incubation was at 30°C until the exponential growth phase was reached. Cells were harvested by centrifugation (4,420 *g*, 4°C, 20 min, Sorvall RC-5B) and the cell pellet was washed and resuspended in NaCl solution (9 g/L). Fermentations were accomplished in glass bottles tightly sealed with rubber septa (90 mL working volume). The bottles were sparged with N_2 prior to and shortly after inoculation. Incubation was at 30°C and 180 rpm (CERTOMAT BS-1).

Sampling and quantitative analysis of sugars and metabolites

Samples of 1.5 mL were frequently removed from yeast fermentations, centrifuged (15,700 *g*, 4°C, 10 min, Centrifuge 5415 R; Eppendorf, Hamburg, Germany) and the supernatant stored at −20°C for HPLC analysis. Cell growth was recorded as increase in OD_{600}. Cell dry weight (CDW) was determined as follows. 10 mL of cell suspension was harvested by centrifugation (3,220 *g*, 4°C, 10 min, Centrifuge 5810 R; Eppendorf), and the cell pellet washed with 10 mL and resuspended in 1 mL NaCl solution (9 g/L). Subsequently, the cell suspension was transferred to pre-dried

(105°C, 12 h) glass vials and then dried until weight constancy (105°C, approximately 12 h). The CDW/OD_{600} correlation was determined to be 0.37 and it was established in triple determination. External fermentation products (ethanol, glycerol, acetate and xylitol) were analyzed by HPLC (Merck-Hitachi LaChrom system, L-7250 autosampler, L-7490 RI detector, L-7400 UV detector; Merck, Whitehouse Station, NJ, USA). The system was equipped with an Aminex HPX-87H column and an Aminex Cation H guard column (both Bio-Rad, Hercules, CA, USA). The operation temperature was 65°C and the flow rate of the mobile phase (5 mM sulfuric acid) was 0.6 mL/h. Carbohydrates (glucose, xylose, arabinose, mannose, galactose and cellobiose) were determined with the same HPLC system but equipped with an Aminex HPX-87P column and a de-ashing guard column (both Bio-Rad). Operation temperature was 80°C for the main column and room temperature for the guard column. The mobile phase was deionized water with a flow rate of 0.4 mL/min.

Data processing and calculations

Reported yield coefficients were always based on mass. Yield coefficients for the xylose phase (Additional files 2 and 4) were calculated for the second phase of the fermentation when glucose was depleted. Carbon balance calculations included metabolite and biomass yields. For the biomass yield a value of 26.4 g/C-mol was utilized [47]. It was further assumed that 1 mol CO_2 was formed per mol acetate and ethanol. q_{Glucose} and q_{Xylose} were calculated by plotting glucose and xylose concentration against fermentation time and fitting the concentration decay with a suitable equation. The first derivative of the respective equation, normalized on the CDW, was used to calculate the uptake rate, which is given in $g/g_{CDW}/h$. Similar to previously published studies [22,40], q_{Xylose} was observed to decrease with reaction time (Additional file 5). Values of q_{Xylose} reported herein are therefore calculated from the initial period of the xylose phase (when glucose was already depleted fully) and represent arithmetic means of at least two determinations made within the first 5 hours of this phase [48]. The courses of q_{Xylose} over time are provided in Additional file 5.

Additional files

Additional file 1: Fermentation of YX media using strains (A) BP10001, (B) IBB10A02 and (C) IBB10B05. Full diamonds, xylose; empty triangles, glycerol; empty squares, xylitol; empty circles, ethanol; crosses, OD_{600}.

Additional file 2: Product yields obtained in fermentations of YX media utilizing strains BP1000, IBB10A02 and IBB10B05.

Additional file 3: 'Glucose phase' of mixed glucose-xylose fermentation in 5% hydrolyzate$_X$. Depicted are the first 8 h of fermentation using strains **(A)** BP10001, **(B)** IBB10A02 and **(C)** IBB10B05. Full time courses are depicted in Figure 2. Full diamonds, xylose; crosses, glucose; empty triangles, glycerol; empty squares, xylitol; empty circles, ethanol.

Additional file 4: Product yields obtained in the 'xylose phase' in fermentations of 5% hydrolyzate$_X$ and 15% hydrolyzate utilizing strains BP10001, IBB10A02 and IBB10B05.

Additional file 5: q_{Xylose} is decreasing with fermentation time. Depicted is the q_{Xylose} over fermentation time in fermentation of **(A)** YX, **(B)** 5% hydrolyzate$_X$ and **(C)** 15% hydrolyzate using strains BP10001 (empty triangles), IBB10A02 (filled squares) and IBB10B05 (filled circles).

Abbreviations

AN: Anaerobic; ATP: Adenosine triphosphate; CDW: Cell dry weight; DM: Dry mass; FPU: filter paper unit; Glc: Glucose; HCD: High cell density; HPLC: High performance liquid chromatography; IUPAC: International Union of Pure and Applied Chemistry; LCD: Low cell density; MM/M-medium: Mineral medium; NADH: Nicotinamide adenine dinucleotide; NADP: Nicotinamide adenine dinucleotide phosphate; NREL: National Renewable Energy Laboratory; OD_{600}: Optical density at 600 nm; Pep: Peptone; PPP: Pentose phosphate pathway; $q_{\mathrm{Xylose/Glucose}}$: Specific xylose/glucose uptake rate; Semi-AN: Semi-anaerobic; SHCF: Separate hydrolysis and co-fermentation; SHF: Separate hydrolysis and fermentation; SSF: Simultaneous saccharification and fermentation; WIS: Water-insoluble content; XDH: Xylitol dehydrogenase; XI: Xylose isomerase; XM-medium: Xylose mineral medium; XR: Xylose reductase; Xyl: Xylose; Xyl/Glc: Xylose/glucose; $Y_{\mathrm{Ethanol/Glycerol/Xylitol/Acetate}}$: Ethanol/glycerol/xylitol/acetate yield; YPD: Yeast extract peptone dextrose; YX: Yeast extract xylose; μ_{Xylose}: Maximum specific anaerobic growth rate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VN, SK, MK and BN designed the research. VN and SK planned the experiments. VN, SK, KL, GM and MW performed the experiments and analyzed data. The manuscript was written from contributions of all authors. VN and BN wrote the paper. All authors read and approved the final manuscript.

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References

1. Chundawat SPS, Beckham GT, Himmel ME, Dale BE: **Deconstruction of lignocellulosic biomass to fuels and chemicals.** *Annu Rev Chem Biomol Eng* 2011, **2**:121–145.
2. Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G: **Bio-ethanol: the fuel of tomorrow from the residues of today.** *Trends Biotechnol* 2006, **24**:549–556.
3. Talebnia F, Karakashev D, Angelidaki I: **Production of bioethanol from wheat straw: an overview on pretreatment, hydrolysis and fermentation.** *Bioresour Technol* 2010, **101**:4744–4753.
4. Palmqvist E, Hahn-Hägerdal B: **Fermentation of lignocellulosic hydrolysates, II: inhibitors and mechanisms of inhibition.** *Bioresour Technol* 2000, **1**:25–33.
5. van Maris A, Abbott D, Bellissimi E, van den Brink J, Kuyper M, Luttik M, Wisselink H, Scheffers W, van Dijken J, Pronk J: **Alcoholic fermentation of carbon sources in biomass hydrolyzates by *Saccharomyces cerevisiae*: current status.** *Antonie Van Leeuwenhoek* 2006, **90**:391–418.
6. Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund M: **Towards industrial pentose-fermenting yeast strains.** *Appl Microbiol Biotechnol* 2007, **74**:937–953.
7. Matsushika A, Inoue H, Kodaki T, Sawayama S: **Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives.** *Appl Microbiol Biotechnol* 2009, **84**:37–53.
8. Sims REH, Mabee W, Saddler JN, Taylor M: **An overview of second generation biofuel technologies.** *Bioresour Technol* 2010, **101**:1570–1580.

9. Brat D, Boles E, Wiedemann B: **Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*.** *Appl Environ Microbiol* 2009, **75:**2304–2311.
10. Ho NW, Chen Z, Brainard AP: **Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose.** *Appl Environ Microbiol* 1998, **64:**1852–1859.
11. Karhumaa K, Garcia Sanchez R, Hahn-Hägerdal B, Gorwa-Grauslund M: **Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*.** *Microb Cell Fact* 2007, **6:**5.
12. Kotter P, Amore R, Hollenberg CP, Ciriacy M: **Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, XYL2, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant.** *Curr Genet* 1990, **18:**493–500.
13. van Maris A, Winkler A, Kuyper M, de Laat W, van Dijken J, Pronk J: **Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component.** *Adv Biochem Eng Biotechnol* 2007, **108:**179–204.
14. Viikari L, Vehmaanperä J, Koivula A: **Lignocellulosic ethanol: from science to industry.** *Biomass Bioenergy* 2012, **46:**13–24.
15. Jeppsson M, Bengtsson O, Franke K, Lee H, Hahn-Hägerdal B, Gorwa-Grauslund MF: **The expression of a *Pichia stipitis* xylose reductase mutant with higher K (M) for NADPH increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*.** *Biotechnol Bioeng* 2006, **93:**665–673.
16. Petschacher B, Nidetzky B: **Engineering *Candida tenuis* xylose reductase for improved utilization of NADH: Antagonistic effects of multiple side chain replacements and performance of site-directed mutants under simulated in vivo conditions.** *Appl Environ Microbiol* 2005, **71:**6390–6393.
17. Matsushika A, Watanabe S, Kodaki T, Makino K, Inoue H, Murakami K, Takimura O, Sawayama S: **Expression of protein engineered NADP + -dependent xylitol dehydrogenase increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*.** *Appl Microbiol Biotechnol* 2008, **81:**243–255.
18. Krahulec S, Klimacek M, Nidetzky B: **Analysis and prediction of the physiological effects of altered coenzyme specificity in xylose reductase and xylitol dehydrogenase during xylose fermentation by *Saccharomyces cerevisiae*.** *J Biotechnol* 2012, **158:**192–202.
19. Krahulec S, Longus K, Klimacek M, Nidetzky B: **On the role of a balanced cofactor recycling between xylose reductase and xylitol dehydrogenase in recombinant xylose fermenting strains of *Saccharomyces cerevisiae*.** *J Biotechnol* 2010, **150:**12.
20. Petschacher B, Nidetzky B: **Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*.** *Microb Cell Fact* 2008, **7:**9.
21. Krahulec S, Klimacek M, Nidetzky B: **Engineering of a matched pair of xylose reductase and xylitol dehydrogenase for xylose fermentation by *Saccharomyces cerevisiae*.** *Biotechnol J* 2009, **4:**684–694.
22. Krahulec S, Petschacher B, Wallner M, Longus K, Klimacek M, Nidetzky B: **Fermentation of mixed glucose-xylose substrates by engineered strains of *Saccharomyces cerevisiae*: role of the coenzyme specificity of xylose reductase, and effect of glucose on xylose utilization.** *Microb Cell Fact* 2010, **9:**16.
23. Demeke M, Dietz H, Li Y, Foulquie-Moreno M, Mutturi S, Deprez S, Den Abt T, Bonini B, Liden G, Dumortier F, Verplaetse A, Boles E, Thevelein JM: **Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolyzates using metabolic and evolutionary engineering.** *Biotechnol Biofuels* 2013, **6:**89.
24. Koppram R, Albers E, Olsson L: **Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass.** *Biotechnol Biofuels* 2012, **5:**32.
25. Sauer U: **Evolutionary engineering of industrially important microbial phenotypes.** In *Metabolic Engineering*, Volume 73. Edited by Nielsen J, Eggeling L, Dynesen J, Gárdonyi M, Gill R, de Graaf A, Hahn-Hägerdal B, Jönsson L, Khosla C, Licari R. Heidelberg: Springer Berlin; 2001:129–169.
26. Sonderegger M, Sauer U: **Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose.** *Appl Environ Microbiol* 2003, **69:**1990–1998.
27. Ha SJ, Galazka JM, Kim SR, Choi JH, Yang X, Seo JH, Glass NL, Cate JH, Jin YS: **Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation.** *Proc Natl Acad Sci USA* 2011, **108:**504–509.
28. Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT: **Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain.** *FEMS Yeast Res* 2005, **5:**925–934.
29. Almeida JRM, Karhumaa K, Bengtsson O, Gorwa-Grauslund M-F: **Screening of *Saccharomyces cerevisiae* strains with respect to anaerobic growth in non-detoxified lignocellulose hydrolyzate.** *Bioresour Technol* 2009, **100:**3674–3677.
30. Kim S, Dale B: **Global potential bioethanol production from wasted crops and crop residues.** *Biomass Bioenergy* 2004, **4:**361–375.
31. Erdei B, Barta Z, Sipos B, Reczey K, Galbe M, Zacchi G: **Ethanol production from mixtures of wheat straw and wheat meal.** *Biotechnol Biofuels* 2010, **3:**16.
32. Olofsson K, Palmqvist B, Liden G: **Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding.** *Biotechnol Biofuels* 2010, **3:**17.
33. Erdei B, Franko B, Galbe M, Zacchi G: **Separate hydrolysis and co-fermentation for improved xylose utilization in integrated ethanol production from wheat meal and wheat straw.** *Biotechnol Biofuels* 2012, **5:**12.
34. Erdei B, Frankó B, Galbe M, Zacchi G: **Glucose and xylose co-fermentation of pretreated wheat straw using mutants of *S. cerevisiae* TMB3400.** *J Biotechnol* 2013, **164:**50–58.
35. Tomas-Pejo E, Oliva J, Ballesteros M, Olsson L: **Comparison of SHF and SSF processes from steam-exploded wheat straw for ethanol production by xylose-fermenting and robust glucose-fermenting *Saccharomyces cerevisiae* strains.** *Biotechnol Bioeng* 2008, **6:**1122–1131.
36. Ballesteros I, Negro MJ, Oliva JM, Cabanas A, Manzanares P, Ballesteros M: **Ethanol production from steam-explosion pretreated wheat straw.** *Appl Biochem Biotechnol* 2006, **129–132:**496–508.
37. Kadam KL, Newman MM: **Development of a low-cost fermentation medium for ethanol production from biomass.** *Appl Microbiol Biotechnol* 1997, **47:**625–629.
38. Jorgensen H: **Effect of nutrients on fermentation of pretreated wheat straw at very high dry matter content by *Saccharomyces cerevisiae*.** *Appl Biochem Biotechnol* 2009, **153:**44–57.
39. Zhou H, Cheng JS, Wang BL, Fink GR, Stephanopoulos G: **Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*.** *Metab Eng* 2012, **14:**611–622.
40. Novy V, Krahulec S, Longus K, Klimacek M, Nidetzky B: **Co-fermentation of hexose and pentose sugars in a spent sulfite liquor matrix with genetically modified *Saccharomyces cerevisiae*.** *Bioresour Technol* 2013, **130:**439–448.
41. Casey E, Sedlak M, Ho NW, Mosier NS: **Effect of acetic acid and pH on the co-fermentation of glucose and xylose to ethanol by a genetically engineered strain of *Saccharomyces cerevisiae*.** *FEMS Yeast Res* 2010, **10:**385–393.
42. Meinander NQ, Boels I, Hahn-Hägerdal B: **Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing XYL1 and XYL2 from *Pichia stipitis* with and without overexpression of TAL1.** *Bioresour Technol* 1999, **68:**79–87.
43. Olofsson K, Rudolf A, Liden G: **Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of *Saccharomyces cerevisiae*.** *J Biotechnol* 2008, **134:**112–120.
44. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D: *Determination of Structural Carbohydrates and Lignin in Biomass*, Technical Report NREL/TP-510-42618. Golden, CO: National Renewable Energy Laboratory; 2011.
45. Esterbauer H, Steiner W, Labudova I, Hermann A, Hayn M: **Production of *Trichoderma* cellulase in laboratory and pilot scale.** *Bioresour Technol* 1991, **36:**51–65.
46. Ghose T: **Measurement of cellulase activities.** *Pure Appl Chem* 1987, **2:**257–268.
47. Lange HC, Heijnen JJ: **Statistical reconciliation of the elemental and molecular biomass composition of *Saccharomyces cerevisiae*.** *Biotechnol Bioeng* 2001, **75:**334–344.
48. Klimacek M, Krahulec S, Sauer U, Nidetzky B: **Limitations in xylose-fermenting *Saccharomyces cerevisiae*, made evident through comprehensive metabolite profiling and thermodynamic analysis.** *Appl Environ Microbiol* 2010, **76:**7566–7574.

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Chapter 2

Supplementary information

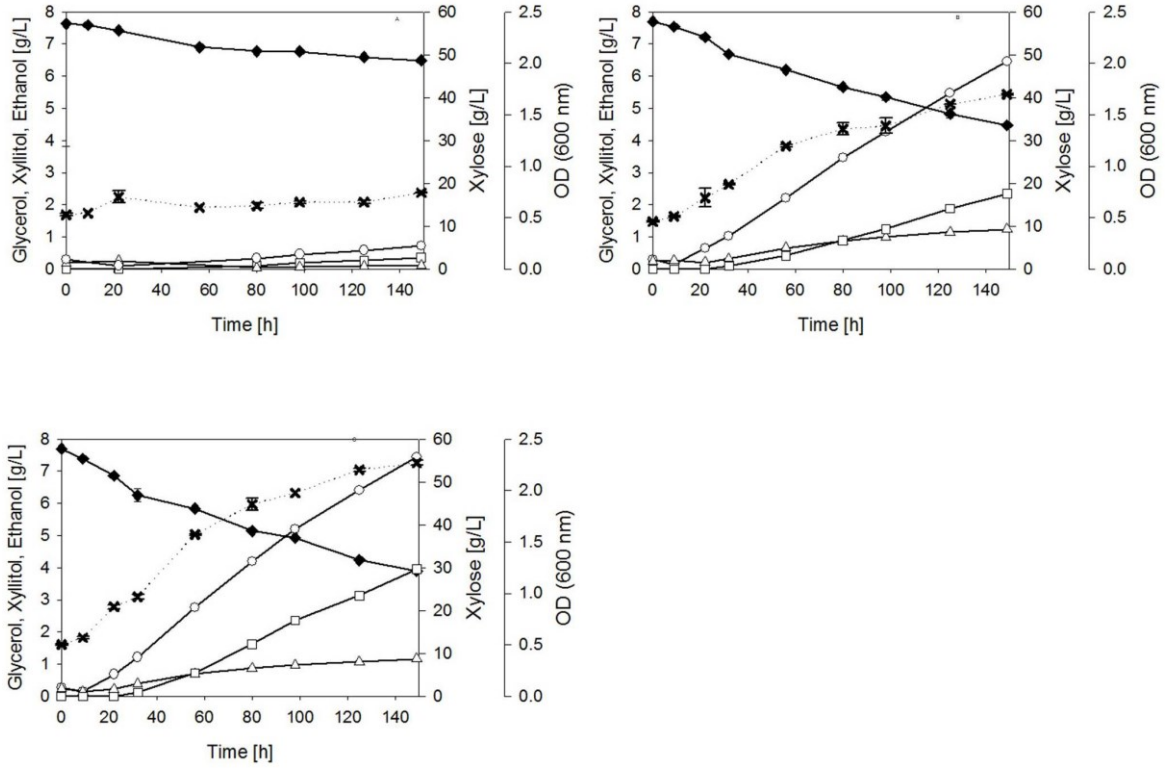
Additional file 1: SI-1
Fermentation of YX media using strains BP10001, IBB10A02 and IBB10B05

Additional file 2: SI-2
Product yields obtained in fermentations of YX media utilizing strains BP10001, IBB10A02 and IBB10B05

Additional file 3: SI-3
'Glucose phase' of mixed glucose-xylose fermentation in 5% hydrolyzate$_X$

Additional file 4: SI-4
Product yields obtained in the 'xylose phase' in fermentations of 5% hydrolyzate$_X$ and 15% hydrolyzate utilizing strains BP10001, IBB10A02 and IBB10B05

Additional file 5: SI-5
q_{Xylose} is decreasing with fermentation time



Additional file 1: Fermentation of YX media using strains (A) BP10001, (B) IBB10A02 and (C) IBB10B05. Full diamonds, xylose; empty triangles, glycerol; empty squares, xylitol; empty circles, ethanol; crosses, OD_{600}.

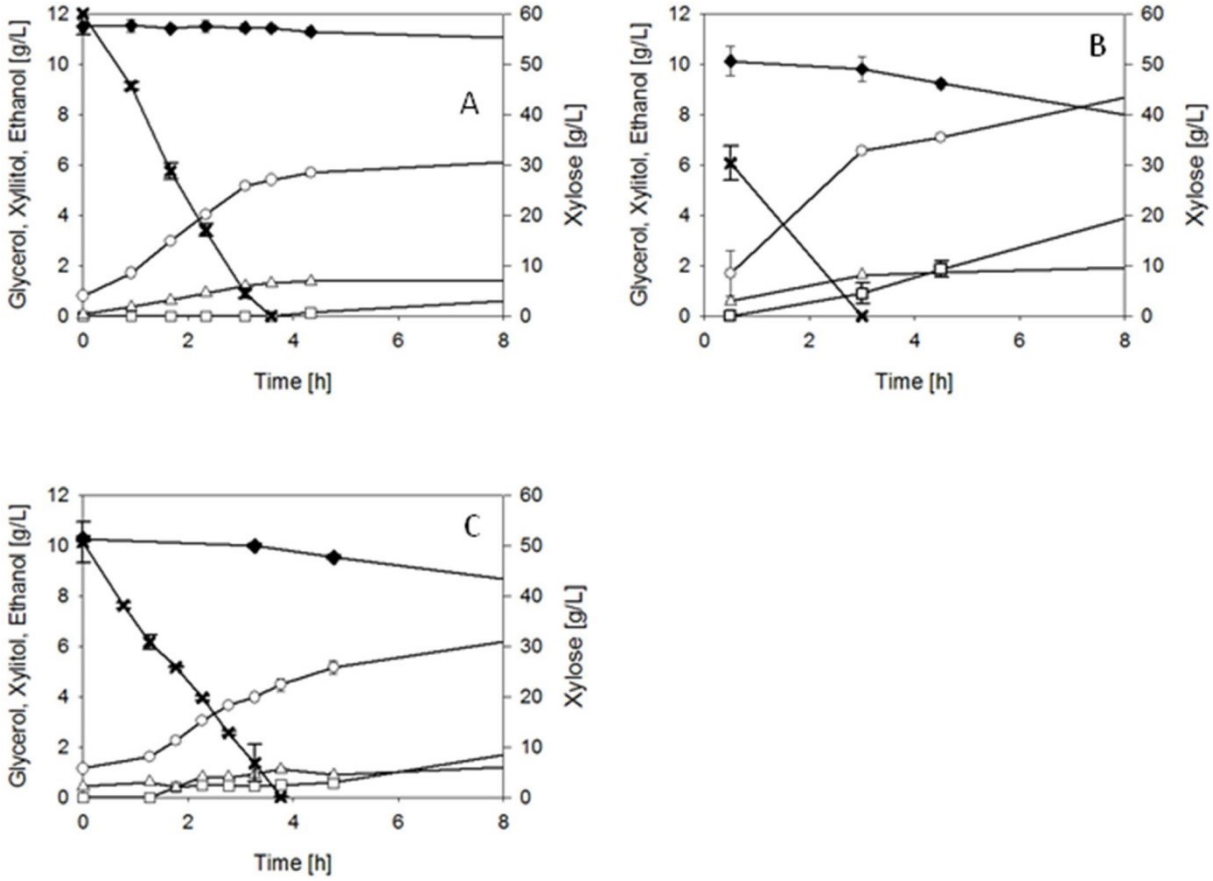
Additional file 2: Product yields obtained in fermentations of YX media utilizing strains BP10001, IBB10A02 and IBB10B05

	BP10001 [a)]	IBB10A02 [b)]	IBB10B05 [b)]
$Y_{Ethanol}$ [g/g_{Xylose}]	0.3	0.33	0.31
$Y_{Glycerol}$ [g/g_{Xylose}]	0.04	0.05	0.04
$Y_{Xylitol}$ [g/g_{Xylose}]	0.16	0.14	0.19
$Y_{Acetate}$ [g/g_{Xylose}]	0.03	0.03	0.03
Y_{BM} [g/g_{Xylose}]	n.d.	0.02	0.02
C-recovery [%]	83	90	90

n.d. - not detectable

a) Due to the small changes in concentration of xylose and metabolites over fermentation time (Additional file 1), data have an increased satistical error.
The yields presented here are mean values of three fermentations and the standard

b) Data was obtained from two independent fermentations. Mean errors were below 10%.



Additional file 3:'Glucose phase' of mixed glucose-xylose fermentation in 5% hydrolyzate$_X$ Depicted are the first 8 h of fermentation using strains **(A)** BP10001, **(B)** IBB10A02 and **(C)** IBB10B05. Full time courses are depicted in Figure 2. Full diamonds, xylose; crosses, glucose; empty triangles, glycerol; empty squares, xylitol; empty circles, ethanol.

Additional file 4: Product yields obtained in the 'xylose phase' in fermentations of 5% hydrolyzate$_X$ and 15% hydrolyzate utilizing strains BP10001, IBB10A02 and IBB10B05.

5%-hydrolyzate$_X$	BP10001 [a)]	IBB10A02 [b)]	IBB10B05 [b)]
$Y_{Ethanol}$ [g/g_{Xylose}]	0.32 ± 0.01	0.31 ± 0.01	0.29 ± 0.01
$Y_{Glycerol}$ [g/g_{Xylose}]	0.05 ± 0.01	0.06 ± 0.01	0.09 ± 0.01
$Y_{Xylitol}$ [g/g_{Xylose}]	0.20 ± 0.02	0.23 ± 0.02	0.18 ± 0.01
$Y_{Acetate}$ [g/g_{Xylose}]	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Y_{BM} [g/g_{Xylose}]	n.d.	n.d.	n.d.
C-recovery [%]	95 ± 2	100 ± 2	93 ± 1

n.d. - not detectable

a) Determined in the first 120 h of fermentation

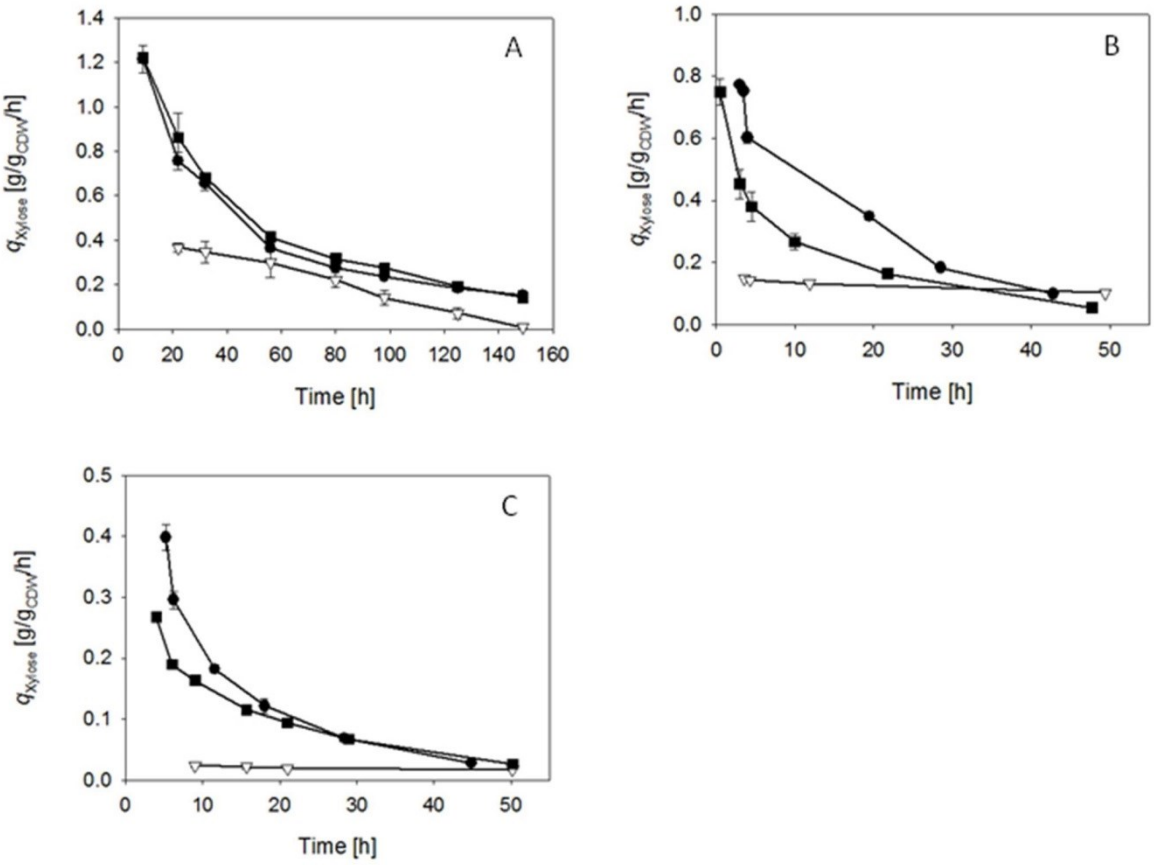
b) Determined in the first 70 h of fermentation

15%-hydrolyzate	BP10001 [a)]	IBB10A02 [b)]	IBB10B05 [b)]
$Y_{Ethanol}$ [g/g_{Xylose}]	0.27 ± 0.02	0.28 ± 0.01	0.31 ± 0.01
$Y_{Glycerol}$ [g/g_{Xylose}]	0.08 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
$Y_{Xylitol}$ [g/g_{Xylose}]	0.30 ± 0.01	0.22 ± 0.01	0.28 ± 0.01
$Y_{acetate}$ [g/g_{Xylose}]	0.09 ± 0.01	0.08 ± 0.01	0.05 ± 0.01
Y_{BM} [g/g_{Xylose}]	n.d.	n.d.	n.d.
C-recovery [%]	105 ± 4	96 ± 1	102 ± 3

n.d. - not detectable

a) Determined in the first 160 h of fermentation

b) Determined in the first 50 h of fermentation



Additional file 5: q_{Xylose} is decreasing with fermentation time. Depicted is the q_{Xylose} over fermentation time in fermentation of (A) YX, (B) 5% hydrolyzate$_X$ and **(C)** 15% hydrolyzate using strains BP10001 (empty triangles), IBB10A02 (filled squares) and IBB10B05 (filled circles).

Chapter 3.1

Towards "homolactic" fermentation of glucose and xylose by engineered *Saccharomyces cerevisiae* harboring a kinetically efficient L-lactate dehydrogenase within *pdc1-pdc5* deletion background

Towards "homolactic" fermentation of glucose and xylose by engineered *Saccharomyces cerevisiae* harboring a kinetically efficient L-lactate dehydrogenase within *pdc1-pdc5* deletion background

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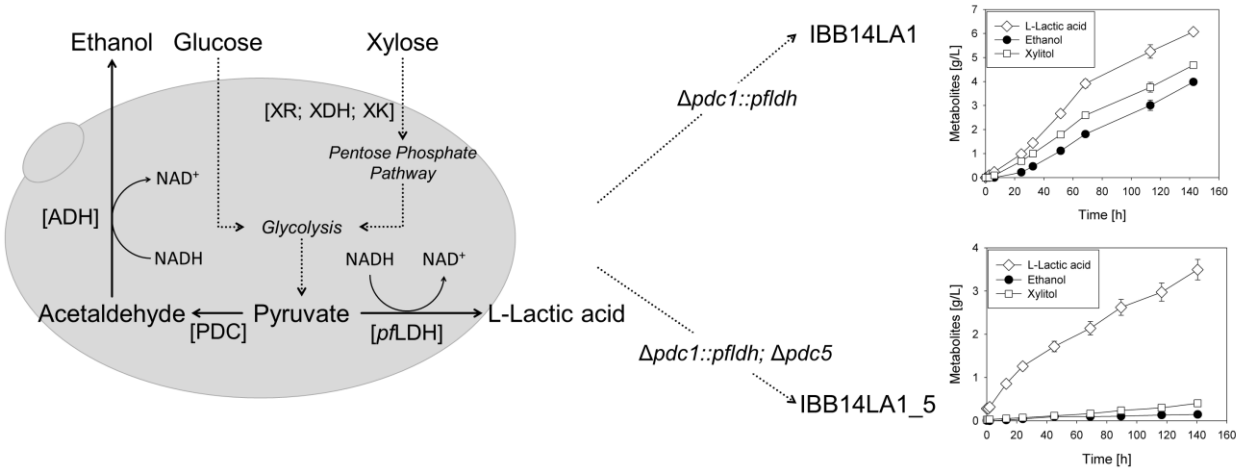
Abbreviations: ADH: Alcohol dehydrogenase; LA: L-Lactic acid; *pf*/*ro*/*lh*/*lc*/*lp*LDH: L-Lactate dehydrogenase from *Plasmodium falciparum*/ *Rhizopus oryzae*/ *Lactobacillus helveticus*/ *L. casei*/ *L. plantarum*; PDC: pyruvate decarboxylase; XDH: Xylitol dehydrogenase; XK: Xylulose kinase; XR: Xylose reductase; YG/X: yeast extract glucose/xylose media; +C/Ac: media supplemented with $CaCO_3$/acetate.

Graphical abstract



Highlights

- Metabolically and evolutionary engineered, XR/XDH containing *S. cerevisiae* strain
- Kinetically efficient L-lactate dehydrogenase from *Plasmodium falciparum* (*pf*LDH)
- Excellent aerobic productivities (~1.8 g/L/h) and yields (0.7 g/g) on glucose
- Anaerobic homofermentative lactic acid production on glucose and xylose

Abstract

L-Lactic acid is an important platform chemical and its production from the lignocellulosic sugars glucose and xylose is therefore of high interest. Tolerance to low pH and a generally high robustness make *Saccharomyces cerevisiae* a promising host for L-lactic acid fermentation but strain development for effective utilization of *both* sugars is an unsolved problem. The herein used *S. cerevisiae* strain IBB10B05 incorporates a NADH-dependent pathway for oxidoreductive xylose assimilation within CEN.PK113-7D background and was additionally evolved for accelerated xylose-to-ethanol fermentation. Selecting the *Plasmodium falciparum* L-lactate dehydrogenase (*pf*LDH) for its high kinetic efficiency, strain IBB14LA1 was derived from IBB10B05 by placing the *pfldh* gene at the *pdc1* locus under control of the *pdc1* promotor. Strain IBB14LA1_5 additionally had the *pdc5* gene disrupted. With both strains, continued L-lactic acid formation from glucose or xylose, each at 50 g/L, necessitated stabilization of pH. Using calcium carbonate (11 g/L), anaerobic shaken bottle fermentations at pH ≥ 5 resulted in L-lactic acid yields (Y_{LA}) of 0.67 g/g glucose and 0.80 g/g xylose for strain IBB14LA1_5. Only little xylitol was formed (≤ 0.08 g/g) and no ethanol. In pH stabilized aerobic conversions of glucose, strain IBB14LA1_5 further showed excellent L-lactic acid productivities (1.8 g/L/h) without losses in Y_{LA} (0.69 g/g glucose). In strain IBB14LA1, the Y_{LA} was lower (≤ 0.18 g/g glucose; ≤ 0.27 g/g xylose) due to ethanol as well as xylitol formation. Therefore, this study shows that a *S. cerevisiae* strain originally optimized for xylose-to-ethanol fermentation was useful to implement L-lactic acid production from glucose and xylose; and with the metabolic engineering strategy applied, advance towards homolactic fermentation of *both* sugars was made.

Keywords: Homolactic; Lactic acid; Lactate dehydrogenase; Xylose fermentation, Anaerobic; *Saccharomyces cerevisiae*

1. Introduction

Environmental concerns and limited fossil resources are driving the development of biorefinery technologies with the aim of gradually replacing petrochemical production of bulk chemicals (Choi et al., 2015). With the rapid increase in annual plastic production, key focus is set on the sustainable manufacturing of precursors for bioplastic polymers (Chen and Patel, 2012), amongst which poly-lactic acid has outstanding potential (Chen and Nielsen, 2016). For large scale biotechnological production of lactic acid, lignocellulose could constitute a cost effective and sustainable feedstock (Chen and Nielsen, 2016). To utilize lignocellulose effectively, this however necessitates the development of a robust fermentation strain capable of efficient conversion of the main lignocellulosic sugars glucose and xylose into lactic acid (Chen and Nielsen, 2016), requirements which are not met by the currently applied microorganisms, e.g. lactic acid bacteria (Abbott et al., 2009b; Benninga, 1990).

Its excellent low-pH tolerance in combination with the high operational stability and the availability of a well-established genetic engineering toolbox made the yeast *Saccharomyces cerevisiae* the chiefly applied host organism for further strain development by metabolic engineering (Abbott et al., 2009b; Chen and Nielsen, 2016; Sauer et al., 2010). Lactic acid production, which is not possible in "natural" strains of *S. cerevisiae*, was achieved through the introduction of a lactate dehydrogenase (LDH), catalyzing the reduction of pyruvate by NADH. However, the resulting strains still showed high ethanol formation and their consequently low lactic acid yield and productivity required further strain engineering to be done (Adachi et al., 1998; Ishida et al., 2005; Sauer et al., 2010; Skory, 2003; Song et al., 2016; Tokuhiro et al., 2009). To channel the metabolic fluxes towards lactic acid formation, there are two metabolic engineering alternatives at the pyruvate branching point. Firstly, by increasing the kinetic pull

from the LDH catalyzed reaction and, secondly, by down regulation or disruption of the competing pyruvate-to-ethanol pathway. Augmentation of the pyruvate-to-lactic acid fluxes was attained by optimization of the *ldh* gene expression (Ishida et al., 2006a; Ishida et al., 2005; Lee et al., 2015), and it was also attempted by utilizing a series of LDHs differing in their catalytic efficiency (Branduardi et al., 2006). However, despite the diverse properties that LDHs from different sources have in regard to kinetics and regulation (Table 1; Garvie (1980), the choice of enzyme for efficient conversion of pyruvate to lactate under *in vivo* boundary conditions has been only sparsely addressed to date (Branduardi et al., 2006; Sauer et al., 2010).

Table 1: Catalytic efficiencies of L-LDHs used for recombinant L-lactic acid production in yeast

	K_M Pyruvate [mM]	K_M NADH [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [a)] [s^{-1} mM^{-1}]	Reference
*pf*LDH	0.03	0.01	450	15000	Gomez et al. (1997)
*ro*LDH[d)]	1.30	-	320	246	Skory et al. (2009)
*lh*LDH[c)]	0.25	-	643	2593	Savijoki and Palva (1997)
*lc*LDH[c)]	1	0.01	5042	5040	Gordon and Doelle (1976)
*lp*LDH	0.5	0.02	5740	11480	Hensel et al. (1977)
*h*LDH-A	0.17	0.01	300	1833	Gomez et al. (1997)

Abbreviations: *pf – Plasmodium falciparum*; *ro – Rhizopus oryzae*; *lh – Lactobacillus helveticus*; *lc – lactobacillus casei*; *lp – Lactobacillus plantarum; h - human*
a) Catalytic efficiency for the reduction of pyruvate to lactic acid
b) Inhibited by fructose-1,6-bisphosphate
c) Dependent on fructose-1,6-bisphosphate

Elimination of the pyruvate-to-ethanol pathway was mainly accomplished by removing one or more genes encoding the pyruvate decarboxylases (PDC). This approach resulted in substantially improved lactic acid yields (Adachi et al., 1998; Ishida et al., 2006b; Skory, 2003), but the PDC deficient strains displayed reduced growth and viability (Pronk et al., 1996; van Maris et al., 2004b).

With the improvement in recombinant glucose-to-lactic acid conversion, strain engineering has to also go one step further and focus on the utilization of hemicellulose-derived pentose sugars (Chen and Nielsen, 2016). To enable xylose-to-lactic acid conversion, various non-conventional yeasts, e.g. *Candida utilis*, *C. sonorensis* and *Pichia stipitis*, which unlike *S. cerevisiae* are naturally capable of xylose utilization, were used as hosts for the strain engineering, which usually settled for just the heterologous expression of LDH (Ilmen et al., 2013; Ilmen et al., 2007; Koivuranta et al., 2014; Tamakawa et al., 2012). An engineered strain of *S. cerevisiae*, enabled to xylose utilization through the integration of the canonical oxidoreductive pathway from the yeasts just mentioned above, in which xylose reductase (XR) and xylitol dehydrogenase (XDH) catalyze a two-step isomerization of xylose into xylulose via xylitol, was also applied for lactic acid production from xylose (Turner et al., 2015; Turner et al., 2016). Remarkably, despite having a fully intact pyruvate-to-ethanol pathway because none of the pyruvate decarboxylase and alcohol dehydrogenase genes had been deleted, the *S. cerevisiae* strain of Turner and colleagues showed a lactic acid yield of up to 0.7 g/g in xylose conversions under aerobic conditions. Under anaerobic conditions or when glucose was utilized instead of xylose, however, mixed ethanol and lactic acid fermentation was obtained (Turner et al., 2015; Turner et al., 2016). In fermentation processes at large scale, generally, the continuous supply of oxygen is undesirable because it significantly adds to process complexity and costs. Despite that, studies on

lactic acid production in recombinant yeasts were mainly performed aerobically (Ilmen et al., 2007; Ishida et al., 2006b; Tamakawa et al., 2012; Turner et al., 2015). Only a few studies conducted anaerobic fermentations, however, with limited success due to low yields (≤ 0.4 g/g) and productivities (≤ 0.1 g/L/h) (Adachi et al., 1998; Koivuranta et al., 2014; Turner et al., 2015). Aim of this study therefore was to develop a *S. cerevisiae* strain capable of efficient L-lactic acid (LA) production from both glucose and xylose under anaerobic conditions. As host, the strain IBB10B05 was chosen. This strain harbors within CEN.PK113-7D genomic background a heterologous XR/XDH pathway, which has the specific feature that due to the engineered XR used, not NADPH, as would be the case when using the wildtype enzyme, but NADH is primarily used for xylose reduction. This offers the advantage that conversion of xylose into xylulose does not create imbalances at the level of the nicotinamide coenzymes in the yeast cell, which affect the distribution of fermentation products formed from xylose. The strain IBB10B05 further comprised a targeted optimization by evolutionary engineering for anaerobic growth during xylose-to-ethanol fermentation (Klimacek et al., 2014). Because of the robust and efficient performance of IBB10B05 under harsh fermentation conditions that included different lignocellulose hydrolyzates (Novy et al., 2013; Novy et al., 2014), this strain was a promising starting point for a metabolic engineering for LA production. To channel metabolic fluxes at the pyruvate branching point towards LA formation, the L-LDH from *Plasmodium falciparum* (*pf*LDH) was utilized. This enzyme was selected for its excellent catalytic efficiency (Table 1) and the fructose-1,6-biphosphate independency of its activity (Berwal et al., 2008; Bzik et al., 1993; Shoemark et al., 2007). Further, the *pf*LDH has a K_M for pyruvate, which is significantly lower than K_M values reported for the PDC enzymes present in *S. cerevisiae* (~2 mM; Agarwal et al. (2013)). The *pfldh* was placed at the *pdc1* locus under control of the native *pdc1* promotor. LA

formation on glucose and xylose was analyzed in a *pdc1* and *pdc1-pdc5* deletion background of IBB10B05.

2. Materials and Methods

2.1. Strains, genes and plasmids

Saccharomyces cerevisiae strain IBB10B05 was used. IBB10B05 has CEN.PK113-7D genomic background and its development was reported elsewhere (Klimacek et al., 2014; Novy et al., 2014; Petschacher and Nidetzky, 2008). The *pfldh* gene (kindly provided by David K. Wilson, University of California, Davis, USA) was cloned into the multiple cloning site of a p427TEF plasmid ("pLA"). The nourseothricin resistance gene (*natR*) was amplified from the pUG74 plasmid (kindly provided by Euroscarf-EUROpean, Institute for Molecular Bioscience, Johann Wolfgang Goethe-University, Frankfurt, Germany).

2.2. Yeast strain construction

In the first step of IBB10B05 strain engineering, the *pdc1* gene was replaced with the *pfldh* gene. The replacement cassette was assembled by PCR, amplifying the *pfldh* gene and the adjacent region coding for the G418 resistance gene (*kanR*) from the "pLA" plasmid. The oligonucleotide primers *integ_fwd* and *integ_rev* were used (Table S1, Supplementary Information). Correct insertion was verified by sequencing and the resulting strain was denoted IBB14LA1. Strain IBB14LA1 was further engineered by replacing the *pdc5* gene with the *natR* gene. The knock-out cassette was amplified from the pUG74 plasmid in a PCR that used *pdc5_k.o._fwd* and *pdc5_k.o._rev* oligonucleotide primers (Table S1). The resulting double-deletion strain was verified by sequencing and denoted IBB14LA1_5. Transformation of yeast strains was performed

with the lithium acetate method (Gietz and Schiestl, 2007). Selected yeast clones were stored in 15% (v/v) glycerol at -80°C.

2.3. Physiological characterization of the different yeast strains

2.3.1. Chemicals and media used

Unless otherwise stated, all chemicals were from Carl Roth + Co KG (Karlsruhe, Germany). Strains IBB10B05 and IBB14LA1 were cultivated in YPD medium containing 10 g/L yeast extract, 20 g/L casein peptone and 20 g/L glucose. YPE medium (10 g/L yeast extract, 20 g/L peptone and 10 g/L ethanol) was used for cultivation of strain IBB14LA1_5. YPD and YPE agar plates contained 15 g/L agar additionally. Aerobic shaken flask and anaerobic shaken bottle fermentations were performed with yeast extract (10 g/L) as sole media supplement. The carbon source was 50 g/L glucose (YG) or xylose (YX). Acetate-supplemented media contained 47.5 g/L of glucose and 2.5 g/L of acetate (YG+Ac). The pH stabilized fermentations were conducted with 11 g/L $CaCO_3$ (YG+C, YG+Ac+C and YX+C). $CaCO_3$ was autoclaved separately and added to the media prior to inoculation.

2.3.2. Measurement of *pf*LDH activity in the crude cell extract

Cells were cultivated aerobically using YPD (IBB14LA1) or YPE (IBB14LA1_5) media in shaken flask cultures. Harvested cells were disrupted mechanically with glass beads in 0.1 M sodium phosphate buffer, pH 7.5, with “cOmplete” protease inhibitor (Roche, Basel, Switzerland) added according recommendations of the supplier. Protein concentration in cell-free extract was measured with the Roti-Quant assay (Roth) referenced against BSA. The *pf*LDH activity was measured by mixing the extract with 1 mM sodium pyruvate and 0.4 mM NADH solution (both in sodium phosphate buffer) and reading the change of absorbance at 340 nm (25°C, DU800 spectrophotometer, Beckman Coulter Inc., Fullerton, USA). Activity is given in

Units (U)/mg total protein in the cell-free extract, with 1 U being defined as the conversion of 1 µmol of NADH per minute. The activity assay was also performed in cell-free extract of the parent strain IBB10B05. Since no drop in NADH concentration was detected, unspecific NADH consumption could be excluded.

2.3.3. Shaken flask und shaken bottle experiments

Seed cultures were prepared with 50 mL YPD (IBB14LA1) or YPE (IBB14LA1_5) medium in 300 mL shaken flasks. Incubation was at 30°C overnight. Cells were used to inoculate 300 mL of YPD or YPE media in 1000 mL baffled shaken flasks to an OD_{600} of 0.05. Incubation was at 30°C until an OD_{600} of 5 was reached. Cells were harvested by centrifugation (4,420 g, 4°C, 20 min, Sorvall RC-5B) and the cell pellet was washed and resuspended in 0.9 % (w/v) NaCl solution. The starting OD_{600} of all experiments was 5.

Aerobic conversions were performed in 300 mL baffled shaken flasks with 50 mL of media. Incubation was at 30°C with gentle agitation (~110 rpm). Anaerobic fermentations were performed in glass bottles with 90 mL working volume. A detailed description can be found in Krahulec et al. (2012), who also confirmed the essentially anoxic reaction conditions of this set up. Incubation was at 30°C and 180 rpm (CERTOMAT BS-1, Sartorius AG, Göttingen, Germany).

2.3.4. Sampling, quantitative analysis of metabolites and data evaluations

The OD_{600} and pH were measured in the cell suspension of the withdrawn samples. The samples were centrifuged (15,700 g, 4°C, 10 min, 5415 R; Eppendorf, Hamburg, Germany) and the supernatant stored at -20°C for HPLC analysis. External fermentation products (lactic acid, ethanol, glycerol, acetic acid, xylitol and pyruvic acid) and sugars (xylose and glucose) were analyzed by HPLC (Merck-Hitachi LaChrom system, L-7250 autosampler, L-7490 RI detector,

L-7400 UV detector; Merck, Whitehouse Station, NJ, USA). The system was equipped with an Aminex HPX-87H column and an Aminex Cation H guard column (both Bio-Rad, Hercules, CA, USA). The operation temperature was 65°C and the flow rate of the mobile phase (5 mM sulfuric acid) was 0.6 mL/min. Reported yield coefficients were based on mass. Carbon balances were calculated with the assumption that 1 mol CO_2 was formed per mol acetate and ethanol. For biomass yields a value of 26.4 g/Cmol was applied (Lange and Heijnen, 2001). Q_{Glc}, Q_{Xyl} and Q_{LA} were calculated by plotting glucose, xylose or LA concentration against fermentation time. The resulting scatter plot was fitted with a suitable equation and the first derivative equals the volumetric uptake and production rate, which is given in g/L/h. Because fermentation rates decrease with reaction time (Novy et al., 2013; Novy et al., 2014), the values of Q_{Glc}, Q_{Xyl} and Q_{LA} reported later represent arithmetic means of the three first sample points.

3. Results

3.1. Verification of *pf*LDH activity in strains IBB14LA1 and IBB14LA1_5

Functional expression of the *pfldh* gene in strains IBB14LA1 and IBB14LA1_5 was verified by measuring *pf*LDH activity in the cell extract of aerobic shaken flask cultivations. Due to the lack of the *pdc1* and *pdc5* genes, biomass growth of strain IBB14LA1_5 on glucose was impaired, as expected (Hohmann, 1991; Pronk et al., 1996), and it was therefore cultivated in YPE medium. Strain IBB14LA1 was cultivated in YPD medium and the *pf*LDH activity was determined to be 1.62 ± 0.14 U/mg protein (N = 3). In strain IBB14LA1_5 a 1.5-fold higher activity of 2.43 ± 0.19 U/mg protein (N = 3) was measured. Since the activity was similar to the recombinant *pf*LDH activity measured in *Escherichia coli* (Berwal et al., 2008; Bzik et al., 1993) and the reported activities of other LDH enzymes expressed in *S. cerevisiae* (Ishida et al., 2005; Skory, 2003;

Turner et al., 2015), we concluded that the *pfldh* was successfully expressed from the *pdc1* promotor in both of the *S. cerevisiae* strains used.

3.2. Characterization of strains IBB14LA1_5 IBB14LA1 in aerobic glucose conversions

Most of the published studies on LA production in engineered yeasts were conducted under aerobic conditions with glucose as carbon source (Sauer et al., 2010). For the sake of comparison, time course analyses of strains IBB14LA1 and IBB14LA1_5 were firstly performed in aerobic shaken flask cultivations on glucose under uncontrolled (YG) and pH stabilized (YG+C) conditions (Figure S1, Supplementary Information). Considering the reported importance of C_2 bodies for growth and viability of *pdc* deficient strains (Flikweert et al., 1999; van Maris et al., 2004b), strain IBB14LA1_5 was also analyzed for glucose conversion in acetate-supplemented media (YG+Ac and YG+Ac+C). The physiological parameters determined from these conversions are summarized in Table S2 (Supplementary Information). A comparison of Y_{LA}, $Y_{Ethanol}$ and Q_{LA} is depicted in Figure 1.

In conversions of glucose under uncontrolled conditions (YG), strain IBB14LA1 consumed the 50 g/L of glucose within 48 h. The final LA titer was 4.8 g/L and the resulting Y_{LA} was 0.10 g/g_{Glc}. Ethanol was the main product with a final titer of 12.2 g/L ($Y_{Ethanol}$ 0.26 g/g_{Glc}). The pH dropped gradually to a value of 3.4 (Figure S1). Conversions were therefore also conducted under pH buffered conditions (YG+C), which stabilized the pH at around 5. As a result the glucose uptake rate (Q_{Glc}) was 1.6-fold increased as compared to the uncontrolled conditions (YG) and the conversion was complete already within 24 h. The final LA titer (10 g/L) and the Y_{LA} were approximately doubled as compared to the uncontrolled conversions (Figure 1). Consequently, ethanol formation was decreased (11 g/L, $Y_{Ethanol}$ 0.23 g/g_{Glc}).

In shaken flask conversions of glucose under uncontrolled conditions, strain IBB14LA1_5 consumed 18.8 g/L of glucose in 48 h, representing a Q_{Glc} of 0.87 g/L/h. LA was the main product (6 g/L), pyruvate ($Y_{Pyruvate}$ 0.17 g/g_{Glc}) was the major by-product, and no ethanol was produced within this time. The pH dropped below 3 (Figure S1). As observed for strain IBB14LA1, addition of $CaCO_3$ improved the conversion rates significantly (YG+C, Figure 1). Thus, Q_{LA} and Q_{Glc} were increased 6.3-fold and 2.8-fold, respectively. The glucose was depleted within 24 h, and ~34 g/L of LA was produced, representing a Y_{LA} of 0.69 g/g_{Glc}. The pH dropped to 3.6. Pyruvate was still the main by-product, but the yield was significantly decreased ($Y_{Pyruvate}$ 0.09 g/g_{Glc}).

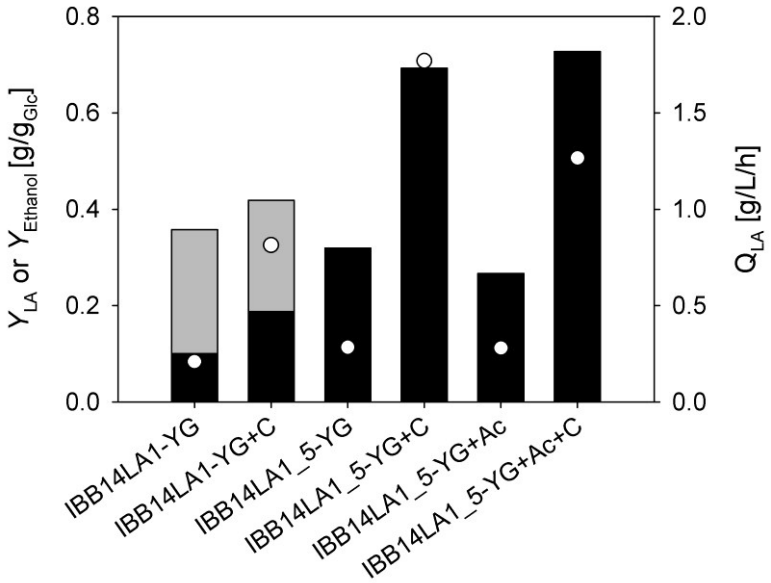


Figure 1: Comparison of Y_{LA} (black bars), $Y_{Ethanol}$ (grey bars) and Q_{LA} (empty circles) in aerobic shaken bottle conversions of 50 g/L glucose (YG) under pH controlled (11 g/L $CaCO_3$; + C) or uncontrolled conditions in the absence and presence of acetate (+ Ac). Depicted are mean values from 2 independent experiments.

Uncontrolled glucose conversions in acetate-supplemented media (YG+Ac) gave highly similar results to experiments in YG media without acetate (Figure 1, Table S2). Under pH stabilized conditions, the addition of acetate resulted in a slightly increased Y_{LA} (0.73 g/g_{Glc}) but the Q_{LA} (~1.3 g/L/h) was decreased 1.4-fold (YG+Ac+C, Figure 1). Based on these results we concluded that acetate was not a critical supplement for LA production from glucose by strain IBB14LA1_5.

Xylose conversion was also analyzed under aerobic conditions (data not shown). The only detectable products with both IBB strains were LA ($Y_{LA} \leq 0.25$ g/g_{Xyl}) and biomass ($Y_{BM} \leq 0.52$ g/g_{Xyl}). In a separate publication we will show that providing low amounts of oxygen (pO_2 ~2 % air) enhances the LA productivity of strain IBB14LA1_5. The results of the current study suggest that xylose, in contrast to glucose, does not lead to repression of respiration in IBB14LA1_5 to the extent necessary for an efficient LA fermentation under aerobic conditions. This behavior of strain IBB14LA1_5 appears consistent with the findings of Jeffries and co-workers who characterized at different levels of the cellular physiology the sugar substrate recognition for ethanol fermentation in xylose-fermenting *S. cerevisiae* (Jin and Jeffries, 2004; Jin et al., 2004). A main conclusion of their study was that xylose, in sharp contrast to glucose, failed to elicit a global "fermentative response" in *S. cerevisiae*. However, since the aim here was to develop a strain capable of converting glucose and xylose to LA under anaerobic conditions, the lack of aerobic xylose conversion was not considered to restrict the relevance of this study.

3.2. Time course analysis for anaerobic fermentations of glucose and xylose by strains IBB14LA1 and IBB14LA1_5

Strain IBB14LA1 was first to be analyzed in anaerobic glucose and xylose fermentations under uncontrolled (YG, YX) and pH stabilized (YG+C, YX+C) conditions. The resulting time courses

are depicted in Figure 2. The physiological parameters determined from the pH stabilized fermentations are summarized in Table 1; those determined from uncontrolled fermentations are summarized in Table S3 (Supplementary Information). Under conditions without pH stabilization, strain IBB14LA1 consumed ~17 g/L of xylose within 140 h (YX, Figure 2, Table S3). The xylose uptake rate (Q_{Xyl}) was 0.16 g/L/h. The final LA titer was ~2.7 g/L and the Y_{LA} was 0.18 g/g_{Xyl}, representing a Q_{LA} of 0.04 g/L/h. Despite the elimination of the *pdc1* gene, a significant amount of ethanol was formed ($Y_{Ethanol}$ 0.20 g/g_{Xyl}). Xylitol was the third major metabolite formed and $Y_{Xylitol}$ was 0.21 g/g_{Xyl} (Table S3).

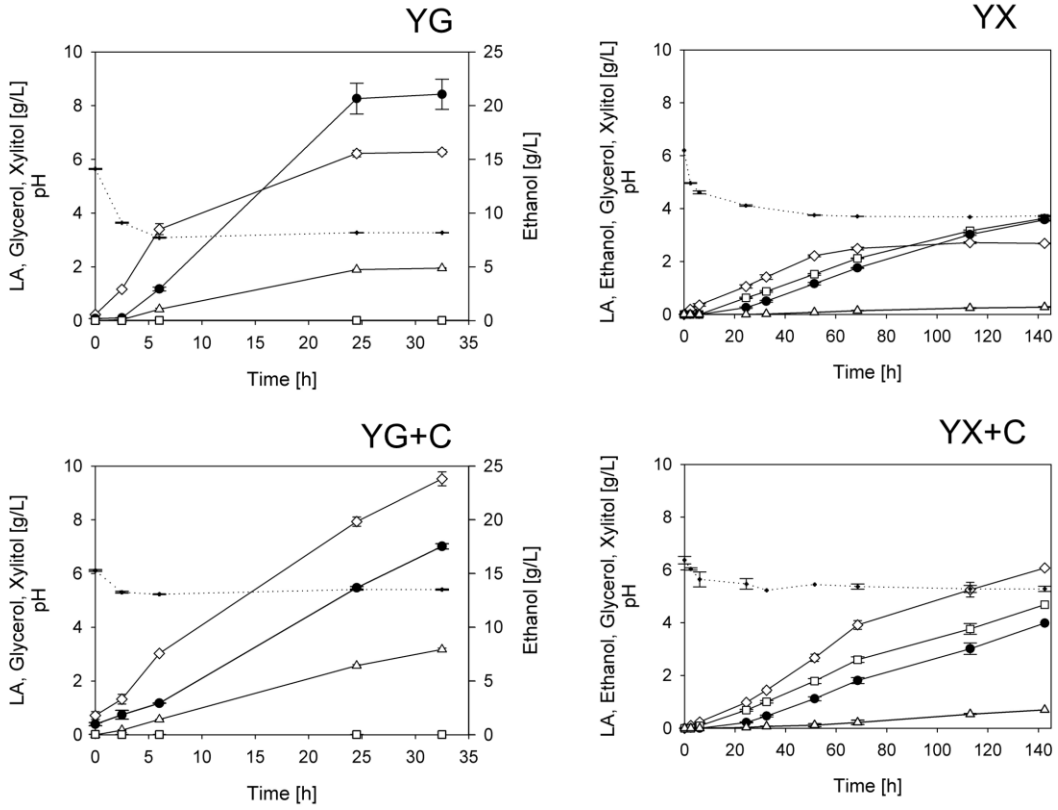


Figure 2: Time courses of anaerobic fermentations by strain IBB14LA1. Reactions were conducted in glucose- (YG and YG+C) and xylose-based (YX and YX+C) media. Addition of $CaCO_3$ is indicated with "+C". Data represent mean values from 2 independent experiments. Symbols: Lactic acid – empty diamonds; ethanol – filled circles; glycerol – empty triangles; xylitol – empty squares; pH – dotted lines.

Addition of $CaCO_3$ to xylose fermentations succeeded in stabilizing the pH at ~5.4 (YX+C, Figure 2). As a result, LA formation was sustained over a longer period of time as compared to the fermentations without added $CaCO_3$. LA was the main product (6 g/L) and Y_{LA} (0.27 g/g_{Xyl})

was improved 1.5-fold. Consequently, less ethanol was formed ($Y_{Ethanol}$ 0.15 g/g_{Xyl}). About 22 g/L of xylose was consumed within 140 h, representing a Q_{Xyl} of 0.20 g/L/h.

Table 2: Physiological parameters of strains IBB14LA1 and IBB14LA1_5 in anaerobic fermentations under pH stabilized conditions

	IBB14LA1		IBB14LA1_5	
	YG+C	YX+C	YG+C	YX+C
$Q_{Glc/Xyl}$ [g/L/h]	2.06 ± 0.01	0.20 ± 0.00	0.07 ± 0.01	0.03 ± 0.01
Q_{LA} [g/L/h]	0.38 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	0.03 ± 0.00
Y_{LA} [g/g]	0.18 ± 0.00	0.27 ± 0.02	0.67 ± 0.04	0.80 ± 0.05
$Y_{Ethanol}$ [g/g]	0.32 ± 0.01	0.15 ± 0.02	0.04 ± 0.01	0.04 ± 0.01
$Y_{Glycerol}$ [g/g]	0.06 ± 0.0	0.02 ± 0.00	0.10 ± 0.00	n.d.
$Y_{Xylitol}$ [g/g]	n.d.	0.19 ± 0.01	0.02 ± 0.00	0.08 ± 0.01
$Y_{Acetate}$ [g/g]	n.d.	0.03 ± 0.01	n.d.	0.04 ± 0.00
$Y_{Pyruvate}$ [g/g]	n.d.	n.d.	n.d.	n.d.
C-recovery[a)] [%]	86.1 ± 1.9	80.7 ± 5.3	90.3 ± 6.1	100.8 ± 7.0

n.d. – not detectable
a) C-recoveries exclude biomass yields because addition of $CaCO_3$ precluded OD_{600} measurement

Under conditions without added $CaCO_3$, glucose conversion was fast for strain IBB14LA1 (Q_{Glc} 2.86 g/L/h). The glucose was consumed within 24 h (YG, Figure 2, Table S3) and 6.3 g/L of LA were produced (Y_{LA} 0.12 g/g_{Glc}). Ethanol was the main product with a final concentration of ~21 g/L ($Y_{Ethanol}$ 0.39 g/g_{Glc}). In pH stabilized glucose fermentations, the final LA titer was 10.8 g/L, representing a Y_{LA} of 0.18 g/g_{Glc} (YG+C, Figure 2, Table 2). Although this presents a ~1.6-fold

improvement in yield as compared to the uncontrolled fermentations, the supplementation of $CaCO_3$ had the additional effects of decreased fermentation rates (Q_{Glc} ~2.0 g/L/h) and increased glycerol formation ($Y_{Glycerol}$ 0.06 g/g_{Glc}).

The results of anaerobic fermentations by strain IBB14LA1_5 are shown in Figure 3. The physiological parameters determined from uncontrolled and pH stabilized fermentations are summarized in Table S3 and Table 2, respectively.

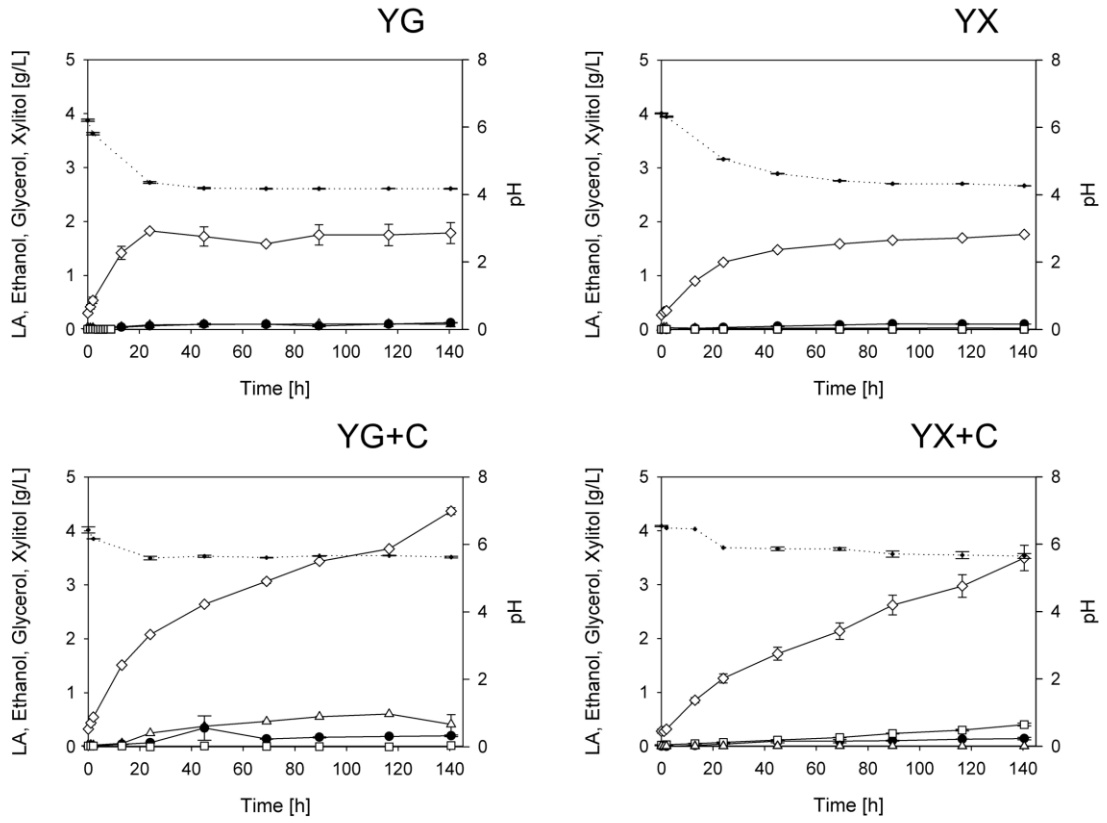


Figure 3: Time courses of anaerobic fermentations by strain IBB14LA1_5. Reactions were conducted in glucose- (YG and YG+C) and xylose-based (YX and YX+C) media. Addition of $CaCO_3$ is indicated with "+C". Data represent mean values from 2 independent experiments. Symbols: Lactic acid – empty diamonds; ethanol – filled circles; glycerol – empty triangles; xylitol – empty squares; pH – dotted lines.

In uncontrolled fermentations, independent of the sugar substrate used, LA was the major product (YG and YX, Figure 3, Table S3). The resulting yields were 0.82 g/g_{Glc} and 0.80 g/g_{Xyl}, respectively, representing ~80% of the theoretical maximum in metabolic yield. Ethanol was only produced in minor amounts (≤ 0.10 g/L) and glycerol ($Y_{Glycerol}$ 0.04 g/g_{Glc}) and xylitol ($Y_{Xylitol}$ 0.05 g/g_{Xyl}) were the main by-products in glucose and xylose fermentations, respectively. The pH dropped to approximately 4. Despite the excellent LA yields, time courses show that LA production stopped after the first 24 h (Figure 3). Under pH stabilized conditions, the LA production progressed well over 140 h of fermentation time. About 3.5 g/L and 4.5 g/L of LA were released within these 140 h in YX+C and YG+C media, respectively. The corresponding Y_{LA} was 67% and 80% of the theoretical maximum (Table 2). Addition of $CaCO_3$ did not influence the distribution of by-products from the xylose fermentation. On glucose, however, glycerol formation was more pronounced ($Y_{Glycerol}$ ~0.10 g/g_{Glc}, Table 2) under the conditions when $CaCO_3$ was present.

4. Discussion

4.1. Advancing towards anaerobic homolactic fermentation of glucose and xylose through a novel strategy of *S. cerevisiae* strain engineering

To approach a large-scale lactic acid biorefinery, strain development has to target at the efficient conversion of the lignocellulose-derived sugars under the most basic process conditions (Chen and Nielsen, 2016; Sauer et al., 2010). Aim of this study therefore was to develop a recombinant strain of *S. cerevisiae* capable of forming LA in high yields from both glucose and xylose, and of doing so under true fermentation conditions, in which oxygen is absent. An engineered yeast strain exhibiting these desired characteristics has, to our knowledge, not been described so far. In this study, a "minimal" metabolic engineering strategy was pursued, which was novel because it employed the background of a *S. cerevisiae* strain originally optimized for robust xylose-to-ethanol fermentation. It further was designed to effectively redirect the flux at the pyruvate branching point not only canonically, by serial *pdc1* and *pdc5* gene deletions, but also by specifically choosing a LDH, from *Plasmodium falciparum* and new in this context, which excelled in its high kinetic efficiency (Table 1). The evidence presented, and its comparison with literature, indicates the importance of individual elements of the metabolic engineering strategy used for an efficient sugar-to-LA conversion in *S. cerevisiae*.

Strain IBB14LA1, which expressed the *pf*LDH gene and had only the *pdc1* gene deleted, showed a Q_{LA} of up to 0.38 g/L/h and 0.06 g/L/h in anaerobic conversions of glucose and xylose, respectively. The Y_{LA} reached between 12% and 27% of the theoretical maximum. The main drawback of IBB14LA1 was high by-product formation, mainly ethanol. The *pdc5* gene was therefore additionally knocked out from the IBB14LA1 genome and the resulting strain IBB14LA1_5 was capable of converting up to 82% of the consumed glucose and xylose to LA

under anaerobic conditions. In aerobic glucose conversions, IBB14LA1_5 achieved a Q_{LA} of ~1.8 g/L/h without substantial loss in Y_{LA} (0.69 g/g_{Glc}). A beneficial impact of increased oxygen supply on Q_{LA} has also been reported for a *pdc1*/*adh1* deficient *S. cerevisiae* strain in the study of Tokuhiro et al. (2009). In contrast to strain IBB14LA1_5, however, the increase in Q_{LA} was accompanied by a decrease in Y_{LA} (Tokuhiro et al., 2009).

In previous studies of LA production by engineered yeasts, the strain evaluation was mostly done aerobically. However, considering the importance for large-scale process operation, the use of truly anaerobic conditions is of particular interest. Anaerobic conversion of glucose was analyzed using *S. cerevisiae* expressing the bovine LDH within wildtype strain background (Adachi et al., 1998). It was also examined using a strain deficient in ethanol and glycerol formation and expressing *h*LDH-A (Ida et al., 2013). In the study of Turner et al. (2015), anaerobic conversion of glucose and xylose was analyzed using a *S. cerevisiae* that harbored the XR/XDH pathway and expressed the *ro*LDH. Anaerobic xylose fermentation was further reported for a *C. sonorensis* strain that expressed a xylose isomerase (converting xylose directly into xylulose) and the *lh*LDH (Koivuranta et al., 2014). Table 3 lists the values of Y_{LA} and Q_{LA} for the different yeast strains, reported directly in the papers or calculated from the data given there, and compares them to the corresponding parameters obtained for strain IBB14LA1_5. Table 3 further states the fermentation performance obtained under aerobic conditions.

In the absence of oxygen, strain IBB14LA1_5 showed the highest Y_{LA} from xylose among the series of strains compared (Table 3). Using glucose as the substrate, the Y_{LA} of strain IBB14LA1_5 was only surpassed by the *S. cerevisiae* strain reported in Ida et al. (2013), which gave the maximum theoretically possible yield of 1.0. Combined disruption of the ethanol and glycerol pathways in the strain of Ida et al. appeared to have suppressed by-product formation

completely. However, the resulting conversion rates were considerably lower as compared to strain IBB14LA1_5 (Table 3). Interestingly, under aerobic conditions the Ida et al. strain showed a sharply deteriorated performance with Y_{LA} of only 0.27 g/g_{Glc} (Ida et al., 2013). It is worth noting therefore that in aerobic glucose conversions, strain IBB14LA1_5 gave the highest Y_{LA} among the strains reported (Table 3). Furthermore, although a direct comparison is difficult due to different biomass concentrations applied in the experiments, strain IBB14LA1 surpassed the other strains regarding the LA production rate (Q_{LA}) from glucose under aerobic conditions (Table 3). Anaerobic conversion of both glucose and xylose for LA production was only described in one study, namely that of Turner et al. (2015). In contrast to strain IBB14LA1_5, strain SR8L lacks manipulation of the *pdc* and *adh* genes. Retention of the native pyruvate-to-ethanol pathway probably explains the relatively high LA production rates of strain SR8L (Table 3). Strain IBB14LA1, which due to the intact *pdc5* gene is still capable of ethanol formation, also showed higher Q_{LA} on both glucose and xylose than strain IBB14LA1_5 (Table 2, Table S3). However, the fermentation performance of strains IBB14LA1 and SR8L was strongly restricted by the low Y_{LA} (Table 2, 3 and S3). s

Table 3: Comparison of Y_{LA} and Q_{LA} in anaerobic glucose or xylose conversions by different engineered yeast strains[a)]

Strain/ Background/ LDH source	Conditions[b)]	Y_{LA} [$g/g_{Glc/Xyl}$]	Q_{LA} [g/L/h]	Reference
S. cerevisiae "SR8L"/ XR, XDH, XK/ *ro*LDH	Glc 40 g/L Xyl 40 g/L OD_{600} 1	0.21 (0.22) 0.43 (0.69)	0.39 (1.32) 0.10 (0.52)	Turner et al. (2015)
C. sonorensis "C29" - / *lh*LDH	Xyl 50 g/L OD_{600} 12	0.56*(0.53)	0.01* (0.16*)	Koivuranta et al. (2014)
S. cerevisiae "Alt2"/ - / bovine LDH	Glc 10 g/L OD_{600} 0.2	0.13 (0.09)	0.07* (0.04*)	Adachi et al. (1998)
*S. cerevisiae "*S252"/ Δ*adh1-5*, Δ*sfa1*, Δ*pdc1*, Δ*gpd1/2* / *h*LDH-A	Glc: 2 g/L OD_{600} 4 (0.1)	1.0 (0.21)	0.01* (0.01*)	Ida et al. (2013)
S. cerevisiae "IBB14LA1_5"/ XR, XDH, XK, Δ*pdc1*, Δ*pdc5*/ *pf*LDH	Glc 50 g/L Xyl 50 g/L OD_{600} 5	0.67 (0.69) 0.80 (n.a.)	0.05 (1.77) 0.03 (n.a.)	*This study*

n.a. – not analyzed; * – values are not directly reported in the paper but are calculated from published data
a) Data from aerobic experiments are in brackets.
b) All data are from experiments involving pH stabilization by $CaCO_3$.

As highlighted by Table 3, strain IBB14LA1_5 features notable overall improvements in LA production under aerobic and anaerobic conditions. Further, IBB14LA1_5 is to date the only

strain capable of glucose and xylose fermentation at yields, which for both sugars are advancing towards homolactic fermentation.

4.2. Use of the kinetically efficient *pf*LDH facilitates high aerobic LA productivities and fast onset of anaerobic LA production

As discussed above, strain IBB14LA1_5 excelled in its high Q_{LA} (1.8 g/L/h) and Y_{LA} (0.7 g/g_{Glc}) during aerobic conversion of glucose. Although accumulation of significant amounts of pyruvate ($\leq$ 3.5 g/L) was observed, mass balance analysis showed that $Y_{Pyruvate}$ was 1.9-fold lower under conditions of stabilized pH as compared to uncontrolled conditions (Table S2). However, when $CaCO_3$ was supplemented for pH stabilization, the Q_{LA} and Q_{Glc} were higher, 6.3-fold and 2.8-fold higher, respectively, as compared to the conversions under uncontrolled conditions. This result clearly demonstrates that the *pf*LDH was able to convert pyruvate very efficiently into LA, even at these high rates of substrate consumption in strain IBB14LA1_5. The importance of a highly efficient *pf*LDH is further highlighted by the fast onset of LA production observed in anaerobic fermentations, independent of the experimental set up or strain used (Figure 2 and 3). In strain IBB14LA1, LA was the predominant product initially and ethanol formation started only after approximately 5 h and 20 h in glucose and xylose fermentations, respectively (Figure 2). In strain IBB14LA1, where the *pf*LDH is in competition with the PDC5 for utilization of pyruvate, the initial "burst" of LA formation was probably facilitated by the much higher catalytic efficiency of the *pf*LDH as compared to the PDC5. Thus, the K_M, k_{cat} and k_{cat}/K_M values for the PDC5 are 2.9 mM, 145 s^{-1} and 71.4 s^{-1} mM^{-1}, respectively (Agarwal et al., 2013). Assuming intracellular pyruvate and NADH concentrations of 0.6 mM and 0.3 mM (Klimacek et al., 2010; Wiebe et al., 2008), respectively, the specific catalytic rate, $v/[E]=k_{cat}\times[S]/(K_M+[S])$, of the *pf*LDH was calculated to be ~418 s^{-1}. This was 17-fold faster than the PDC5 catalyzed reaction

(~25 s^{-1}). This indicates that a large increase in the concentration of PDC5 relative to the concentration of *pf*LDH would be needed, to enable pyruvate-to-ethanol conversion to compete strongly with LA formation.

Evidence that ethanol was formed by strain IBB14LA1 on glucose as well as on xylose, delayed in time but nonetheless, might be ascribed to the autoregulative nature of the *pdc* gene expression. Lack of the PDC1 protein was shown in earlier studies of *S. cerevisiae* to promote an enhanced expression of the *pdc5* gene (Eberhardt et al., 1999; Hohmann and Cederberg, 1990). This effect may have allowed the PDC activity in strain IBB14LA1 to catch up with the LDH activity, compensating low enzyme efficiency by high enzyme amount. It is interesting that, when xylose was used as carbon source, strain IBB14LA1 did not only show a later onset of ethanol formation (~20 h) as compared to conversions of glucose (~5 h; Figure 2). The xylose fermentation also exhibited a 1.5-fold higher Y_{LA} than the corresponding glucose fermentation, irrespective of whether $CaCO_3$ was supplemented for pH control (Table 2, Table S3). A higher Y_{LA} on xylose as compared to glucose was also observed by Turner et al. (2015). To explain effect of sugar substrate on LA yield, the authors suggested that the slower rate of xylose as compared to glucose utilization could have resulted in comparably lower levels of pyruvate in the cell. Based on the smaller K_M of the *ro*LDH as compared to the PDC, this would favor the LDH over the PDC catalyzed reaction, resulting in increased Y_{LA} and a decreased $Y_{Ethanol}$ (Turner et al., 2015). Based on the kinetic arguments for strain IBB14LA1 presented above, however, it seems extremely unlikely that, at a constant ratio of *pf*LDH and PDC5 in the cell, the change in the intracellular pyruvate concentration could have influenced the relative specific rates of the two enzymes to the extent of which Y_{LA} was affected by ethanol formation

Since besides pyruvate the *pf*LDH also requires NADH for activity, the specific rate of LA formation might as well be limited by the coenzyme. The study of Lee et al. (2015) suggested that enhancement of the NADH availability due to perturbation of the intracellular NADH/NAD^+ ratio could be useful to improve LA production from glucose by *S. cerevisiae*. However, the NADH K_M of *pf*LDH is only 0.01 mM, suggesting that the specific catalytic rate of the enzyme should be rather insensitive, even to a 10-fold decrease in the NADH concentration (0.3 mM → 0.03 mM).

Since limitations at the level of the *specific* rate of *pf*LDH are unlikely, the observed change in LA and ethanol formation on glucose and xylose probably arose due to variation in LDH/PDC enzyme amount in dependence on the sugar substrate consumed. Supported by the much later onset of ethanol formation in xylose as compared to glucose fermentations (Table 2), it is likely that xylose affects the *pdc* gene expression not as strongly as glucose does. Differential gene expression on xylose as compared to glucose in *S. cerevisiae* has been described before (Jin and Jeffries, 2004; Jin et al., 2004), the autoregulative *pdc* gene expression, however, has not been included in these studies.

4.3. Continued anaerobic LA formation at controlled pH – the importance of a robust strain background

In anaerobic sugar conversions by strains IBB14LA1 and IBB14LA1_5 without pH stabilization, the LA formation stopped after about 50 h and 20 h, respectively. As shown in Figures 2 and 3, the LA-time-curve mirrored the pH-time-curve and the LA production ceased when the pH reached the pK_a of LA (~3.8; Sauer et al. (2010)). The deceleration of LA formation in uncontrolled conversions was probably caused by accumulation of undissociated LA in the fermentation media. The protonated form of the acid can freely diffuse into the cell, where the

neutral pH in the cytosol leads to dissociation. To prevent acidification, an ATP dependent proton export is initiated (Thomas et al., 2002). It has been further suggested that LA production in *S. cereviae* does not yield net ATP and that the demand of ATP for the export of LA is the likely reason for it (Abbott et al., 2009a; van Maris et al., 2004a; van Maris et al., 2004b). This implies that a homofermentative strain of *S. cerevisiae* would be incapable of meeting the ATP requirements for maintenance under anaerobic conditions (Abbott et al., 2009a; van Maris et al., 2004b). The halt in anaerobic fermentations without pH stabilization using the *pdc1-pdc5* deficient strain IBB14LA1_5 was therefore likely a result of ATP depletion in the cell, caused by the energy intensive out-ward pumping of protons as well as lactate export (Abbott et al., 2009a; Thomas et al., 2002; van Maris et al., 2004b). In support of this, Song et al. (2016) have recently shown that LA productivities in ethanol-deficient strains of *S. cerevisiae* can be improved by introducing a recombinant ATP-independent pathway for acetyl-CoA production from acetaldehyde. Manipulation of the acetyl-CoA pathway has been identified as useful engineering strategy for establishing efficient cell factories (Nielsen, 2014). In this study, however, strain IBB14LA1_5 was capable of continued LA production over 140 h of fermentation when the pH was stabilized, with yields approaching "homolactic" fermentation on glucose and also xylose. We believe that this excellent performance of strain IBB14LA1_5 must be ascribed to a large extent to the already high robustness of the evolved progenitor strain IBB10B05. Following laboratory evolution, strain IBB10B05 was capable of efficient mixed glucose-xylose fermentation even under the harsh conditions presented by technological substrates such as sulfite liquor and lignocellulose hydrolyzates (Novy et al., 2013; Novy et al., 2014). Therefore, strains of *S. cerevisiae* developed for effective alcoholic fermentation might generally be useful starting points for metabolic engineering for LA production. The capability of strain IBB14LA1_5 is

underlined by its high efficiency of aerobic glucose conversion. Despite the comparably low $CaCO_3$ concentration of 11 g/L used in our experiments (cf. (Ishida et al., 2006b; Koivuranta et al., 2014; Turner et al., 2015; Turner et al., 2016), which made the pH drop to a value as low as 3.6 during the reactions, strain IBB14LA1_5 could still convert 80% of the offered glucose into LA (Figure S1). By way of comparison, Ilmen et al. (2013) studied the impact of varying $CaCO_3$ concentrations on the conversion efficiency of a recombinant C. *sonorensis* strain. When a similar $CaCO_3$ concentration was used as in the study here, the *C. sonorensis* strain showed a low Y_{LA} of only ~0.35 $g/g_{Glc.}$

5. Conclusions

To advance homolactic fermentation on lignocellulose-derived glucose and xylose, this study presents for the first time a *S. cerevisiae* strain capable of converting both sugars at high Y_{LA} (≥ 0.7 g/g). By choosing a robust xylose-fermenting strain background, the *pdc1-pdc5* deficient strain IBB14LA1_5 was capable of continued LA formation under anaerobic conditions provided that the pH was stabilized with added $CaCO_3$. Selection of the kinetically efficient *pf*LDH appeared to have been decisive for high LA yields, high aerobic conversion rates and fast onset of anaerobic LA production. This study therefore shows that by applying a suitable metabolic engineering strategy, *pdc*-deletion strains of *S. cerevisiae* can constitute promising hosts for anaerobic LA production on glucose and xylose.

References

Abbott, D. A., van den Brink, J., Minneboo, I. M., Pronk, J. T., van Maris, A. J. 2009a. Anaerobic homolactate fermentation with *Saccharomyces cerevisiae* results in depletion of ATP and impaired metabolic activity. FEMS Yeast Res 9:349-357.

Abbott, D. A., Zelle, R. M., Pronk, J. T., van Maris, A. J. A. 2009b. Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges. FEMS Yeast Res 9:1123-1136.

Adachi, E., Torigoe, M., Sugiyama, M., Nikawa, J.-I., Shimizu, K. 1998. Modification of metabolic pathways of *Saccharomyces cerevisiae* by the expression of lactate dehydrogenase and deletion of pyruvate decarboxylase genes for the lactic acid fermentation at low pH value. J Ferment Bioeng 86:284-289.

Agarwal, P. K., Uppada, V., Noronha, S. B. 2013. Comparison of pyruvate decarboxylases from *Saccharomyces cerevisiae* and *Komagataella pastoris* (*Pichia pastoris*). Appl Microbiol Biotechnol 97:9439-9449.

Benninga, H. 1990. A history of lactic acid making. The Netherlands:Springer. 478.

Berwal, R., Gopalan, N., Chandel, K., Prasad, G. B., Prakash, S. 2008. *Plasmodium falciparum*: enhanced soluble expression, purification and biochemical characterization of lactate dehydrogenase. Exp Parasitol 120:135-141.

Branduardi, P., Sauer, M., De Gioia, L., Zampella, G., Valli, M., Mattanovich, D., Porro, D. 2006. Lactate production yield from engineered yeasts is dependent from the host background, the lactate dehydrogenase source and the lactate export. Microb Cell Fact 5:4.

Bzik, D. J., Fox, B. A., Gonyer, K. 1993. Expression of *Plasmodium falciparum* lactate dehydrogenase in *Escherichia coli*. Mol Biochem Parasitol 59:155-166.

Chen, G. Q., Patel, M. K. 2012. Plastics derived from biological sources: present and future: a technical and environmental review. Chem Rev 112:2082-2099.

Chen, Y., Nielsen, J. 2016. Biobased organic acids production by metabolically engineered microorganisms. Curr Opin Biotechnol 37:165-172.

Choi, S., Song, C. W., Shin, J. H., Lee, S. Y. 2015. Biorefineries for the production of top building block chemicals and their derivatives. Metab Eng 28:223-239.

Eberhardt, I., Cederberg, H., Li, H., König, S., Jordan, F., Hohmann, S. 1999. Autoregulation of yeast pyruvate decarboxylase gene expression requires the enzyme but not its catalytic activity. Eur J Biochem 262:191-201.

Flikweert, M. T., de Swaaf, M., van Dijken, J. P., Pronk, J. T. 1999. Growth requirements of pyruvate-decarboxylase-negative *Saccharomyces cerevisiae*. FEMS Microbiol Lett 174:73-79.

Garvie, E. I. 1980. Bacterial lactate dehydrogenases. Microbiol Rev 44:106-139.

Gietz, R. D., Schiestl, R. H. 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2:31-34.

Gomez, M. S., Piper, R. C., Hunsaker, L. A., Royer, R. E., Deck, L. M., Makler, M. T., Vander Jagt, D. L. 1997. Substrate and cofactor specificity and selective inhibition of lactate dehydrogenase from the malarial parasite *P. falciparum*. Mol Biochem Parasitol 90:235-246.

Gordon, G. L., Doelle, H. W. 1976. Purification, properties and immunological relationship of L(+)-Lactate Dehydrogenase from *Lactobacillus casei*. Eur J Biochem 67:543-555.

Hensel, R., Mayr, U., Fujiki, H., Kandler, O. 1977. Comparative studies of lactate dehydrogenases in lactic acid bacteria. Amino-acid composition of an active-site region and chemical properties of the L-lactate dehydrogenase of *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, and *Lactobacillus acidophilus*. Eur J Biochem 80:83-92.

Hohmann, S. 1991. Characterization of PDC6, a third structural gene for pyruvate decarboxylase in *Saccharomyces cerevisiae*. J Bacteriol 173:1963-1969.

Hohmann, S., Cederberg, H. 1990. Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes PDC1 and PDC5. Eur J Biochem 188:615-621.

Ida, Y., Hirasawa, T., Furusawa, C., Shimizu, H. 2013. Utilization of *Saccharomyces cerevisiae* recombinant strain incapable of both ethanol and glycerol biosynthesis for anaerobic bioproduction. Appl Microbiol Biotechnol 97:4811-4819.

Ilmen, M., Koivuranta, K., Ruohonen, L., Rajgarhia, V., Suominen, P., Penttila, M. 2013. Production of L-lactic acid by the yeast *Candida sonorensis* expressing heterologous bacterial and fungal lactate dehydrogenases. Microb Cell Fact 12:53.

Ilmen, M., Koivuranta, K., Ruohonen, L., Suominen, P., Penttila, M. 2007. Efficient production of L-lactic acid from xylose by *Pichia stipitis*. Appl Environ Microbiol 73:117-123.

Ishida, N., Saitoh, S., Ohnishi, T., Tokuhiro, K., Nagamori, E., Kitamoto, K., Takahashi, H. 2006a. Metabolic engineering of *Saccharomyces cerevisiae* for efficient production of pure L(+)-lactic acid. Appl Biochem Biotechnol 131:795-807.

Ishida, N., Saitoh, S., Onishi, T., Tokuhiro, K., Nagamori, E., Kitamoto, K., Takahashi, H. 2006b. The effect of pyruvate decarboxylase gene knockout in *Saccharomyces cerevisiae* on L-lactic acid production. Biosci, Biotechnol, Biochem 70:1148-1153.

Ishida, N., Saitoh, S., Tokuhiro, K., Nagamori, E., Matsuyama, T., Kitamoto, K., Takahashi, H. 2005. Efficient production of L-lactic acid by metabolically engineered *Saccharomyces cerevisiae* with a genome-integrated L-lactate dehydrogenase gene. Appl Environ Microbiol 71:1964-1970.

Jin, Y. S., Jeffries, T. W. 2004. Stoichiometric network constraints on xylose metabolism by recombinant *Saccharomyces cerevisiae*. Metab Eng 6:229-238.

Jin, Y. S., Laplaza, J. M., Jeffries, T. W. 2004. *Saccharomyces cerevisiae* engineered for xylose metabolism exhibits a respiratory response. Appl Environ Microbiol 70:6816-6825.

Klimacek, M., Kirl, E., Krahulec, S., Longus, K., Novy, V., Nidetzky, B. 2014. Stepwise metabolic adaption from pure metabolization to balanced anaerobic growth on xylose explored for recombinant *Saccharomyces cerevisiae*. Microb Cell Fact 13:37.

Klimacek, M., Krahulec, S., Sauer, U., Nidetzky, B. 2010. Limitations in xylose-fermenting *Saccharomyces cerevisiae*, made evident through comprehensive metabolite profiling and thermodynamic analysis. Appl Environ Microbiol 76:7566-7574.

Koivuranta, K., Ilmen, M., Wiebe, M., Ruohonen, L., Suominen, P., Penttila, M. 2014. L-lactic acid production from D-xylose with *Candida sonorensis* expressing a heterologous lactate dehydrogenase encoding gene. Microb Cell Fact 13:107.

Krahulec, S., Kratzer, R., Longus, K., Nidetzky, B. 2012. Comparison of *Scheffersomyces stipitis* strains CBS 5773 and CBS 6054 with regard to their xylose metabolism: implications for xylose fermentation. MicrobiologyOpen 1:64-70.

Lange, H. C., Heijnen, J. J. 2001. Statistical reconciliation of the elemental and molecular biomass composition of *Saccharomyces cerevisiae*. Biotechnol Bioeng 75:334-344.

Lee, J. Y., Kang, C. D., Lee, S. H., Park, Y. K., Cho, K. M. 2015. Engineering cellular redox balance in *Saccharomyces cerevisiae* for improved production of L-lactic acid. Biotechnol Bioeng 112:751-758.

Nielsen, J. 2014. Synthetic biology for engineering acetyl coenzyme A metabolism in yeast. MBio 5:

Novy, V., Krahulec, S., Longus, K., Klimacek, M., Nidetzky, B. 2013. Co-fermentation of hexose and pentose sugars in a spent sulfite liquor matrix with genetically modified *Saccharomyces cerevisiae*. Bioresour Technol 130:439-448.

Novy, V., Krahulec, S., Wegleiter, M., Muller, G., Longus, K., Klimacek, M., Nidetzky, B. 2014. Process intensification through microbial strain evolution: mixed glucose-xylose fermentation in wheat straw hydrolyzates by three generations of recombinant *Saccharomyces cerevisiae*. Biotechnol Biofuels 7:49.

Petschacher, B., Nidetzky, B. 2008. Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. Microb Cell Fact 7:9.

Pronk, J. T., Yde Steensma, H., Van Dijken, J. P. 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12:1607-1633.

Sauer, M., Porro, D., Mattanovich, D., Branduardi, P. 2010. 16 years research on lactic acid production with yeast - ready for the market? Biotechnol Genet Eng Rev 27:229-256.

Savijoki, K., Palva, A. 1997. Molecular genetic characterization of the L-lactate dehydrogenase gene (ldhL) of *Lactobacillus helveticu*s and biochemical characterization of the enzyme. Appl Environ Microbiol 63:2850-2856.

Shoemark, D. K., Cliff, M. J., Sessions, R. B., Clarke, A. R. 2007. Enzymatic properties of the lactate dehydrogenase enzyme from *Plasmodium falciparum*. FEBS J 274:2738-2748.

Skory, C. D. 2003. Lactic acid production by *Saccharomyces cerevisiae* expressing a *Rhizopus oryzae* lactate dehydrogenase gene. J Ind Microbiol Biotechnol 30:22-27.

Skory, C. D., Mertens, J. A., Rich, J. O. 2009. Inhibition of *Rhizopus* lactate dehydrogenase by fructose 1,6-bisphosphate. Enzyme Microb Technol 44:242-247.

Song, J. Y., Park, J. S., Kang, C. D., Cho, H. Y., Yang, D., Lee, S., Cho, K. M. 2016. Introduction of a bacterial acetyl-CoA synthesis pathway improves lactic acid production in *Saccharomyces cerevisiae*. Metab Eng 35:38-45.

Tamakawa, H., Ikushima, S., Yoshida, S. 2012. Efficient production of L-lactic acid from xylose by a recombinant *Candida utilis* strain. J Biosci Bioeng 113:73-75.

Thomas, K. C., Hynes, S. H., Ingledew, W. M. 2002. Influence of medium buffering capacity on inhibition of *Saccharomyces cerevisiae* growth by acetic and lactic acids. Appl Environ Microbiol 68:1616-1623.

Tokuhiro, K., Ishida, N., Nagamori, E., Saitoh, S., Onishi, T., Kondo, A., Takahashi, H. 2009. Double mutation of the PDC1 and ADH1 genes improves lactate production in the yeast *Saccharomyces cerevisiae* expressing the bovine lactate dehydrogenase gene. Appl Microbiol Biotechnol 82:883-890.

Turner, T. L., Zhang, G. C., Kim, S. R., Subramaniam, V., Steffen, D., Skory, C. D., Jang, J. Y., Yu, B. J., Jin, Y. S. 2015. Lactic acid production from xylose by engineered *Saccharomyces cerevisiae* without PDC or ADH deletion. Appl Microbiol Biotechnol 99:8023-8033.

Turner, T. L., Zhang, G. C., Oh, E. J., Subramaniam, V., Adiputra, A., Subramaniam, V., Skory, C. D., Jang, J. Y., Yu, B. J., Park, I., Jin, Y. S. 2016. Lactic acid production from cellobiose and xylose by engineered *Saccharomyces cerevisiae*. Biotechnol Bioeng 113:1075-1083.

van Maris, A. J., Konings, W. N., van Dijken, J. P., Pronk, J. T. 2004a. Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. Metab Eng 6:245-255.

van Maris, A. J. A., Winkler, A. A., Porro, D., van Dijken, J. P., Pronk, J. T. 2004b. Homofermentative lactate production cannot sustain anaerobic growth of engineered *Saccharomyces cerevisiae*: possible consequence of energy-dependent lactate export. Appl Environ Microbiol 70:2898-2905.

Wiebe, M. G., Rintala, E., Tamminen, A., Simolin, H., Salusjärvi, L., Toivari, M., Kokkonen, J. T., Kiuru, J., Ketola, R. A., Jouhten, P., Huuskonen, A., Maaheimo, H., Ruohonen, L., Penttilä, M. 2008. Central carbon metabolism of Saccharomyces cerevisiae in anaerobic, oxygen-limited and fully aerobic steady-state conditions and following a shift to anaerobic conditions. FEMS Yeast Res 8:140-154.

Chapter 3.1

Supplementary information

Supplementary Table S1: SI-1
Sequences of oligonucleotides used in this study

Supplementary Table S2: SI-2
Physiological parameters of strains IBB14LA1 and IBB14LA1_5 in aerobic shaken flask cultivations

Supplementary Table S3: SI-3
Physiological parameters of strains IBB14LA1 and IBB14LA1_5 in anaerobic glucose and xylose fermentations under uncontrolled conditions

Supplementary Figure S4: SI-4
Aerobic shaken flaks cultivations of strain IBB14LA1 and IBB14LA1_5 under uncontrolled and pH stabilized conditions

Supplementary Table S1: Sequences of oligonucleotides used in this study[a)]

Primer	Sequence (5'-3')
ldh_integ_fwd	TCTACTCATAACCTCACGCAAAATAACACAGTCAAATCAATCAAA *ATGGCACCAAAAGCAA*
ldh_integ_rev	AAAAATGCTTATAAAACTTTAACTAATAATTAGAGATTAAATCGC *CTCCTTGACAGTCTTGACG*
pdc5_k.o._fwd	TCAATCTCAAAGAGAACAACACAATACAATAACAAGAAGAACAA AT*AGGTCTAGAGATCT*
pdc5_k.o._rev	AAAAAAATACACAAACGTTGAATCATGAGTTTTATGTTAATTAGC *ATTAAGGGTTCTCGAGA*

a) Underlined: region homologues to the target region; bold: restriction sites

Supplementary Table S2: Physiological parameters of strains IBB14LA1 and IBB14LA1_5 in aerobic shaken flask cultivations

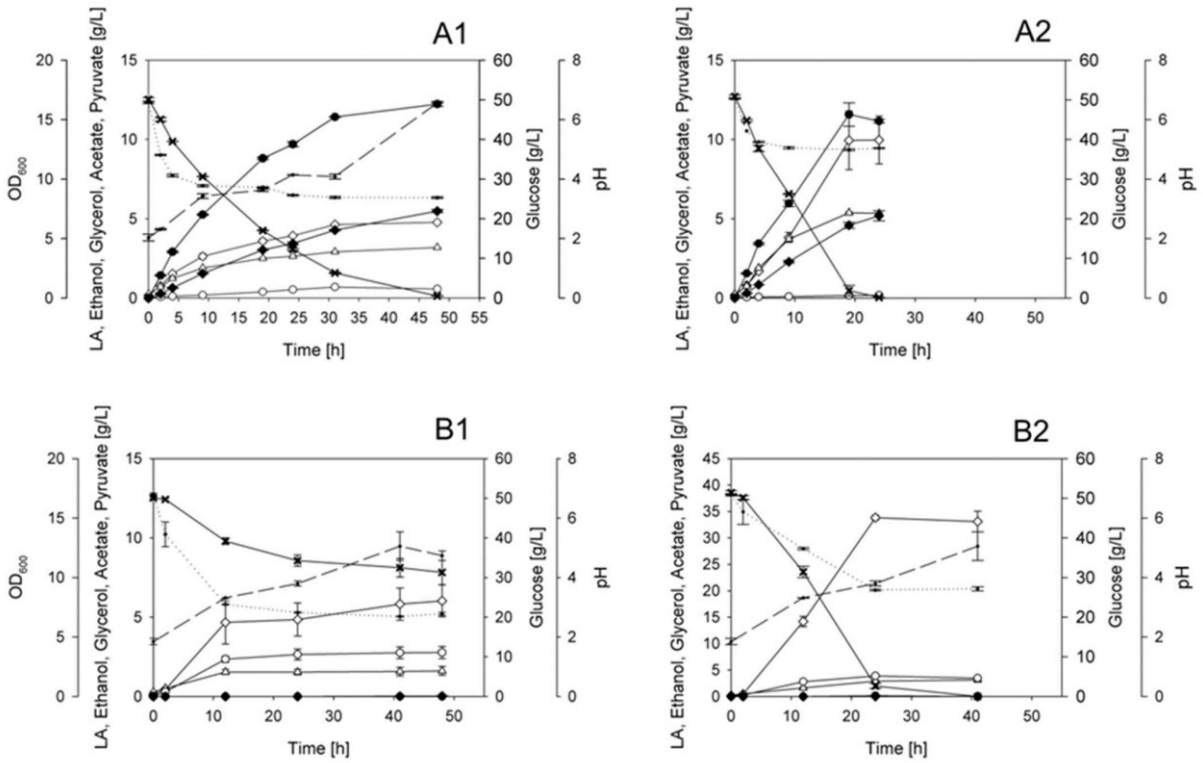
	IBB14LA1		IBB14LA1_5			
	YG[b)]	YG+C[b)]	YG[b)]	YG+C[b)]	YG+Ac	YG+Ac+C
Q_{Glc} [g/L/h]	2.01 ± 0.04	3.30 ± 0.14	0.87 ± 0.06	2.47 ± 0.05	0.79 ± 0.01	1.73 ± 0.01
Q_{LA} [g/L/h]	0.21 ± 0.01	0.87 ± 0.18	0.28 ± 0.07	1.77 ± 0.04	0.28 ± 0.09	1.27 ± 0.02
Y_{LA} [g/g]	0.10 ± 0.00	0.19 ± 0.03	0.32 ± 0.03	0.69 ± 0.01	0.27 ± 0.05	0.73 ± 0.02
$Y_{Ethanol}$ [g/g]	0.26 ± 0.01	0.23 ± 0.00	n.d.	n.d.	n.d.	n.d.
$Y_{Glycerol}$ [g/g]	0.06 ± 0.00	0.11 ± 0.01	0.08 ± 0.02	0.06 ± 0.00	0.03 ± 0.02	0.03 ± 0.00
$Y_{Xylitol}$ [g/g]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$Y_{Acetate}$ [g/g]	0.10 ± 0.01	0.10 ± 0.01	n.d.	n.d.	n.a.	n.a.
$Y_{Pyruvate}$ [g/g]	0.01 ± 0.00	n.d.	0.17 ± 0.04	0.09 ± 0.00	0.25 ± 0.04	0.11 ± 0.01
Y_{BM} [g/g]	0.09 ± 0.00	n.a.	0.20 ± 0.02	n.a.	0.15 ± 0.01	n.a.
C-recovery[c)] [%]	92.2 ± 1.2	89.2 ± 2.1	78.5 ± 5.6	83.7 ± 0.8	75.5 ± 5.7	87.1 ± 0.5

n.d. – not detectable; n.a. – not analyzed
a) Parameters were determined for the first 48 h of conversion, or when the glucose was depleted
b) Time courses are depicted in Supplementary Figure S1
c) C-recoveries of $CaCO_3$ supplemented fermentations ("+C") exclude biomass yields

Supplementary Table S3: Physiological parameters of strains IBB14LA1 and IBB14LA1_5 in anaerobic glucose and xylose fermentations under uncontrolled conditions

	IBB14LA1		IBB14LA1_5	
	YG	YX	YG	YX
$Q_{Glc/Xyl}$ [g/L/h]	2.86 ± 0.02	0.16 ± 0.00	n.d.	n.d.
Q_{LA} [g/L/h]	0.30 ± 0.01	0.04 ± 0.01	n.d.	n.d.
Y_{LA} [g/g]	0.12 ± 0.00	0.18 ± 0.01	0.82 ± 0.02	0.80 ± 0.08
$Y_{Ethanol}$ [g/g]	0.39 ± 0.03	0.20 ± 0.02	0.054 ± 0.01	0.04 ± 0.01
$Y_{Glycerol}$ [g/g]	0.04 ± 0.0	0.02 ± 0.00	0.04 ± 0.00	n.d.
$Y_{Xylitol}$ [g/g]	n.d.	0.21 ± 0.02	0.02 ± 0.00	0.05 ± 0.00
$Y_{Acetate}$ [g/g]	n.d.	n.d.	0.03 ± 0.00	0.03 ± 0.00
$Y_{Pyruvate}$ [g/g]	n.d.	n.d.	n.d.	n.d.
C-recovery [%]	91.3 ± 4.8	78.9 ± 5.3	90.3 ± 6.07	99.2 ± 10.9

n.d. – not detectable; n.a. – not analyzed



Supplementary Figure S4: Aerobic shaken flaks cultivations of strain IBB14LA1 (panels A-1/2) and IBB14LA1_5 (panels B-1/2) under uncontrolled (panels A/B-1) and pH stabilized (A/B-2) conditions. Data represent mean values from 2 experiments. Symbols: Glucose – crosses; lactic acid – empty diamonds; ethanol – filled circles; glycerol – empty triangles; xylitol – empty squares; acetate – filled diamonds; pyruvate – empty circles; OD_{600} – dashed lines; pH – dotted lines.

Chapter 3.2

Micro-aeration improves L-lactic acid yields and productivities on xylose by a *pdc1-pdc5* deficient *Saccharomyces cerevisiae* strain

Rapid Communication

Micro-aeration improves L-lactic acid yields and productivities on xylose by a *pdc1-pdc5* deficient *Saccharomyces cerevisiae* strain

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Abbreviations: LA: L-Lactic acid; *pf*LDH: L-Lactate dehydrogenase from *Plasmodium falciparum*; PDC: pyruvate decarboxylase; YPE: yeast extract peptone ethanol media; YX: yeast extract xylose media.

Abstract

To advance towards a lactic acid biorefinery, strain development has to target at efficient and robust conversion of the lignocellulose-derived sugar xylose. We have recently presented a xylose-fermenting *Saccharomyces cerevisiae* strain expressing the L-lactate dehydrogenase from *Plasmodium falciparum* in a *pdc1-pdc5* deletion background. Under anaerobic conditions, strain IBB14LA1_5 converted glucose and xylose to L-lactic acid at high yields (Y_{LA} ≥0.7 g/g), but showed low productivities (Q_{LA} ≤0.07 g/L/h). Providing oxygen to glucose conversions significantly increased Q_{LA} (1.8 g/L/h) at constant Y_{LA} (0.7 g/g), but decreased efficiencies on xylose (Y_{LA} 0.15 g/g; Q_{LA} 0.04 g/L/h). With the aim of accelerating xylose-to-lactic acid conversions in strain IBB14LA1_5, experiments in this study were conducted under microaerophilic conditions (pO_2 2 % air), which increased Q_{LA} (0.11 g/L/h) significantly. The Y_{LA} (≤0.38 g/g) was higher as compared to fully aerobic conditions, partially recovering the high Y_{LA} obtained in anaerobic conversions. Comparison of anaerobic, aerobic and microaerophilic conversions suggested that xylose, unlike glucose, does not repress the respiratory response in lactic acid producing *S. cerevisiae*. This study shows that elucidation of the role of oxygen in xylose-to-lactic acid conversions is essential for process optimization, which is required to render large scale lactic acid production feasible.

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1 Introduction

Lactic acid is an industrially important bulk chemical and has received increasing attention as a precursor for the production of the bioplastic polymer poly-lactic acid [1-3]. To meet the growing demand sustainably and environmentally compatible, production of lactic acid from renewable resources, ideally from lignocellulosic waste streams, must be facilitated [2]. This necessitates the development of a robust fermentation organism capable of efficient conversion of the lignocellulose-derived sugars glucose and xylose [1-4], requirements which are not met by native lactic acid producing organisms [1-3]. Based on the pH tolerance and the operational stability, *Saccharomyces cerevisiae* was the preferred host strain [1, 3], but efficient conversion of glucose and xylose still remains an unsolved problem [1, 3, 5]. To aim at this bottleneck, we have recently developed the L-lactic acid (LA) producing *S. cerevisiae* strain IBB14LA1_5 [6]. It is a descendant of strain IBB10B05, which harbors the xylose reductase and xylitol dehydrogenase catalyzed xylose assimilation pathway. Strain IBB10B05 was further evolutionary engineered for acceleration of xylose-to-ethanol fermentation [7]. Strain IBB14LA1_5 was derived from strain IBB10B05 by introduction of the L-lactate dehydrogenase from *Plasmodium falciparum*, an enzyme selected for its excellent kinetic properties (k_{cat}/K_M for pyruvate 15000 s^{-1} mM^{-1} [8]). The *pfldh* gene was placed at the *pdc1* locus under control of the native *pdc1* promotor. By removing the *pdc5* gene, the pyruvate-to-ethanol pathway was disrupted [6]. Under anaerobic conditions, strain IBB14LA1_5 showed excellent LA yields (Y_{LA}) on glucose and xylose (>0.7 g/g), but LA productivities (Q_{LA}) did not exceed 0.07 g/L/h [6]. Reasons were considered to be found in insufficient ATP production and the resulting decrease in cell viability [6, 9]. Consequently, providing oxygen increased Q_{LA} 26-fold to ~1.8 g/L/h in glucose conversions, without losses in Y_{LA} [6]. On xylose however, aerobic conditions did not increase Q_{LA} but significantly decreased Y_{LA} (*this study*; [6]).

The aim of this study was to increase LA productivities in xylose conversions by providing microaerophilic conditions. Further the role of oxygen in xylose-to-LA conversions was elucidated by comparing the performance of strain IBB14LA1_5 in anaerobic, fully aerobic and microaerophilic conversion experiments.

2 Materials and methods

2.1 Strain and media

The *S. cerevisiae* strain IBB14LA1_5 (*pdc1*::*pfldh*, *Δpdc5*) was utilized and a detailed description of strain construction has been published elsewhere [6]. Seed and starter cultures were prepared in YPE media (10 g/L yeast extract, 20 g/L peptone from casein, 10 g/L ethanol). Conversion experiments were conducted in YX media (10 g/L yeast extract, 50 g/L xylose). All chemicals were from Carl Roth + Co KG (Karlsruhe, Germany).

2.2 Anaerobic and aerobic cultivations

Anaerobic fermentations were performed in glass bottles with 90 mL working volume. A detailed description of this setup can be found in Krahulec et al. (2012), who also confirmed the essentially anaerobic conditions [10]. Incubation was at 30°C and 190 rpm (CERTOMAT BS-1, Sartorius AG, Göttingen, Germany). Aerobic conversions were conducted in 300 mL baffled shaken flasks filled with 50 mL media and closed with cotton foam stoppers. Incubation was at 30°C and ~110 rpm. Starting OD_{600} was 5 in all experiments. The laboratory procedure has been described in detail elsewhere [6].

2.2.1 Microaerophilic bioreactor fermentations

Microaerophilic conversions were conducted in 2 L Labfors III bioreactors (Infors AG, Bottmingen, Switzerland), equipped with off-gas ethanol and CO_2 analyzers (Innova 1313, LumaSense Technologies A/S, Frankfurt am Main, Germany). The conversions were run in two phases. An aerobic biomass production phase with YPE media (1 L working volume) and a microaerophilic conversion phase with YX media (2 L). The conditions were 30°C

and pH 6.8, continuously adjusted with 5 M NaOH. The dissolved oxygen concentration (pO_2) was 50 % for the biomass production phase and 2 % for the conversion phase. pO_2 was controlled online with an agitation (100 to 1000 rpm) and aeration (0.16 to 2 L/min pressurized air) cascade. Starter cultures were prepared in YPE medium and added to the bioreactor to an OD_{600} of 0.5. When an OD_{600} of ~10 was reached, the conversion phase was initiated by adding 1 L of YX media and reducing the pO_2 to 2%.

2.3. Sampling, analysis of metabolites and data evaluation

Samples were taken from all cultivations, centrifuged and the supernatant used for HPLC analysis of xylose and metabolites (LA, ethanol, xylitol, glycerol, acetate and pyruvate). Reported yield coefficients were based on mass. For carbon recoveries it was assumed that 1 mol CO_2 was formed per mol acetate and ethanol. For biomass yields a value of 26.4 g/Cmol was applied [11]. Q_{Xyl} and Q_{LA} are given in g/L/h. A detailed description of the HPLC setup and the data evaluation can be found elsewhere [6].

3 Results and Discussion

3.1 Xylose-to-LA conversions under different aeration conditions

Time course analysis of strain IBB14LA1_5 in xylose-to-LA conversions under anaerobic, aerobic and microaerophilic conditions was performed, and results are depicted in Figure 1. The physiological parameters are summarized in Table 1. Under anaerobic conditions, ~3.5 g/L of LA was produced in 140 h of fermentation, corresponding to a Y_{LA} of 0.80 g/g_{Xyl} and a Q_{LA} of 0.03 g/L/h. The main by-product was xylitol ($Y_{Xylitol}$ 0.08 g/g_{Xyl}) (Table 1 and [6]). In aerobic cultivations, strain IBB14LA1_5 produced 1 g/L LA in 24 h of fermentation. Since LA started to decrease again (Figure 1), cultivations were stopped after 42 h. Under aerobic conditions, *S. cerevisiae* is able to take up and metabolize lactic acid [12]. The Y_{LA} determined for the first 24 h of conversion was 0.15 g/g_{Xyl}. The main product was biomass, and the high biomass yield ($Y_{Biomass}$ 0.44 g/g_{Xyl}) shows that shaken

flask cultivations provided fully aerobic conditions [13]. In microaerophilic xylose conversions, 7.1 g/L LA were produced in 140 h of fermentation time. As it can be seen in Figure 1, the reaction proceeded in 2 phases. A transition phase ("phase I"; 0 to 27 h) where a residual amount of ethanol (2.8 g/L) was still present and a second phase ("phase II"; 27 to 140 h) in which xylose was the only remaining carbon source (Table 1, Figure 1). In phase I, Q_{LA} was fastest (Q_{LA} ~0.11 g/L/h) and biomass growth was observed ($Y_{Biomass}$ 0.31 g/g_{Xyl}). Y_{LA} based on xylose and ethanol was 0.24 $g/g_{Xyl+Ethanol}$. The Y_{LA} based on consumed xylose only was ~0.33 g/g_{Xyl}. One of the primarily formed by-products was acetate ($Y_{Acetate}$ 0.18 g/gXyl), which is an intermediate of ethanol catabolism. In phase II (27 to 140 h), the conversion slowed down (Q_{Xylose} ~0.04 g/L/h) and the Y_{LA} was ~0.38 g/g_{Xylose}. Carbon was majorly lost in CO_2 production (Y_{CO2} 0.58 g/g_{Xyl}).

3.2 Oxygen supply for optimization of LA yields and productivities

In glucose-to-LA conversions by strain IBB14LA1_5, the Q_{LA} increased from 0.03 g/L/h to 1.8 g/L/h at constant Y_{LA} (0.7 g/g_{Glc}), when cultivations were conducted under aerobic as compared an anaerobic conditions [6]. On xylose, however, changing from anaerobic to aerobic conversions did not show an improvement in Q_{LA} but decreased Y_{LA} 5.3-fold (Table 1). Supported by the the high biomass yield, it seems likely that under fully aerobic conditions, fluxes at the pyruvate branching point are channeled away from LA formation towards the respiratory pathways. This observation is in line with metabolic flux analyses in xylose-to-ethanol converting *S. cerevisiae* strains, where it was shown that xylose does not repress respiratory responses in the same way as glucose does [14]. To still use oxygen as boost for fermentation rates, but reduce the metabolic "pull" towards the TCA cycle, the process set up was changed. Online regulation of the bioreactors facilitated adjustment of low oxygen levels (pO_2 2% air) without changing the experimental setup e.g. the starting OD_{600}. Under microaerophilic conditions, strain IBB14LA1_5 showed an up to

2.8-fold increased Q_{LA} (phase 1, Table 1) as compared to aerobic and anaerobic conditions. The Y_{LA} was ~2.5-fold increased, recovering the high Y_{LA} observed in anaerobic fermentations to some extent (Table 1). Interestingly, the xylitol yield in microaerophilic conversions was 4-fold lower as compared to anaerobic conditions, despite the 4- to 10-fold increased xylose conversion rate (Phase I and II, Table 1). Oxygen probably helped to resolve redox imbalances by NADH oxidation by the electron transport chain [13]. Since pyruvate-to-LA reduction is NADH-dependent, an increased pool of NADH further can be useful to improve Y_{LA} [15]. Potential optimization of ethanol production by adjusting aeration has been also been suggested in the study of Jin and Jeffries [13].

4 Concluding remarks

This study shows that low amounts of oxygen can facilitate continuous LA production over 140 h of cultivation by the *pdc1-pdc5* deficient *S. cerevisiae* strain IBB14LA1_5. The Q_{LA} (≤ 0.11 g/L/h) was up to 2.8-fold improved as compared to anaerobic conditions. Results of strain performances in aerobic, anaerobic and microaerophilic conversions suggest that xylose, in contrast to glucose, does not suppress the respiratory response in strain IBB14LA1_5. Oxygen seems to be essential for high xylose-to-LA conversion efficiencies and thus, represents a clear target for strain development and process optimization.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

5 References

[1] Abbott, D. A., Zelle, R. M., Pronk, J. T., van Maris, A. J. A., Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges. *FEMS Yeast Res* 2009, *9*, 1123-1136.

[2] Chen, Y., Nielsen, J., Biobased organic acids production by metabolically engineered microorganisms. *Curr Opin Biotechnol* 2016, *37*, 165-172.

[3] Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., 16 years research on lactic acid production with yeast - ready for the market? *Biotechnol. Genet. Eng. Rev.* 2010, *27*, 229-256.

[4] Pang, X., Zhuang, X., Tang, Z., Chen, X., Polylactic acid (PLA): research, development and industrialization. *Biotechnol J* 2010, *5*, 1125-1136.

[5] Turner, T. L., Zhang, G. C., Kim, S. R., Subramaniam, V.*, et al.*, Lactic acid production from xylose by engineered *Saccharomyces cerevisiae* without PDC or ADH deletion. *Appl Microbiol Biotechnol* 2015.

[6] Novy, V., Brunner, B., Müller, G., Nidetzky, B., Expression of a kinetically efficient L-lactic dehydrogenase in *pdc1* and *pdc1-pdc5* deletion backgrounds of xylose-fermenting *Saccharomyces cerevisiae*: implications for homofermentative conversion of glucose and xylose to L-lactic acid. *In submission*. 2016.

[7] Klimacek, M., Kirl, E., Krahulec, S., Longus, K.*, et al.*, Stepwise metabolic adaption from pure metabolization to balanced anaerobic growth on xylose explored for recombinant *Saccharomyces cerevisiae*. *Microb Cell Fact* 2014, *13*, 37.

[8] Gomez, M. S., Piper, R. C., Hunsaker, L. A., Royer, R. E.*, et al.*, Substrate and cofactor specificity and selective inhibition of lactate dehydrogenase from the malarial parasite *P. falciparum*. *Mol Biochem Parasitol* 1997, *90*, 235-246.

[9] van Maris, A. J. A., Winkler, A. A., Porro, D., van Dijken, J. P., Pronk, J. T., Homofermentative lactate production cannot sustain anaerobic growth of engineered *Saccharomyces cerevisiae*: possible consequence of energy-dependent lactate export. *Appl Environ Microbiol* 2004, *70*, 2898-2905.

[10] Krahulec, S., Kratzer, R., Longus, K., Nidetzky, B., Comparison of *Scheffersomyces stipitis* strains CBS 5773 and CBS 6054 with regard to their xylose metabolism: implications for xylose fermentation. *MicrobiologyOpen* 2012, *1*, 64-70.

[11] Lange, H. C., Heijnen, J. J., Statistical reconciliation of the elemental and molecular biomass composition of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* 2001, *75*, 334-344.

[12] Lodi, T., Guiard, B., Complex transcriptional regulation of the *Saccharomyces cerevisiae* CYB2 gene encoding cytochrome b2: CYP1(HAP1) activator binds to the CYB2 upstream activation site UAS1-B2. *Mol Cell Biol* 1991, *11*, 3762-3772.

[13] Jin, Y. S., Jeffries, T. W., Stoichiometric network constraints on xylose metabolism by recombinant *Saccharomyces cerevisiae*. *Metab Eng* 2004, *6*, 229-238.

[14] Jin, Y.-S., Laplaza, J. M., Jeffries, T. W., *Saccharomyces cerevisiae* engineered for xylose metabolism exhibits a respiratory response. *Appl Environ Microbiol* 2004, *70*, 6816-6825.

[15] Lee, J. Y., Kang, C. D., Lee, S. H., Park, Y. K., Cho, K. M., Engineering cellular redox balance in *Saccharomyces cerevisiae* for improved production of L-lactic acid. *Biotechnol Bioeng* 2015, *112*, 751-758.

Tables and Figures

Table 1. Physiological parameters of strain IBB14LA1_5 in anaerobic, aerobic and microaerophilic xylose-to-LA conversion experiments[a)]

Conditions	anaerobic	aerobic	microaerophilic	
c_{Xylose} [g/L][b)]	4.0	6.3	15.5	
c_{LA} [g/L][b)]	3.5	1.0	7.1	
			Phase I (0-27h)	*Phase II* (27-143h)
Q_{Xyl} [g/L/h]	0.03 ± 0.01	0.21 ± 0.01	0.31 ± 0.07	0.12 ± 0.00
Q_{LA} [g/L/h]	0.03 ± 0.00	0.04 ± 0.01	0.11 ± 0.00	0.04 ± 0.01
Y_{LA} [g/g_{Xyl}]	0.80 ± 0.05	0.15 ± 0.03	0.24 ± 0.04[c)]/0.33 ± 0.03	0.38 ± 0.01
$Y_{Ethanol}$ [g/g_{Xyl}]	0.04 ± 0.01	n.d.	n.d.	n.d.
$Y_{Glycerol}$ [g/g_{Xyl}]	n.d.	n.d.	n.d.	n.d.
$Y_{Xylitol}$ [g/g_{Xyl}]	0.08 ± 0.01	n.d.	n.d.	0.02 ± 0.00
$Y_{Acetate}$ [g/g_{Xyl}]	0.04 ± 0.01	n.d.	0.18 ± 0.04[c)]	0.10 ± 0.01
$Y_{Biomass}$ [g/g_{Xyl}]	n.d.	0.44 ± 0.01	0.31 ± 0.03[c)]	n.d.
Y_{CO2} [g/g_{Xyl}]	n.a.[d)]	n.a.	0.25 ± 0.02[c)]	0.58 ± 0.01
C-recovery [%]	100.8 ± 7.0	63 ± 6.5	94.6 ± 2.2[c)]	99.6 ± 4.9

n.d. – not detectable; n.a. – not analyzed

a) Time courses are depicted in Figure 1. Time frame of data analysis was 24 for the aerobic and 140 h for the anaerobic and microaerophilic conversion experiments. Data for the anaerobic fermentations were taken from [6]

b) Consumed xylose and final LA titers

c) Yields and mass balance are calculated on consumed xylose and ethanol

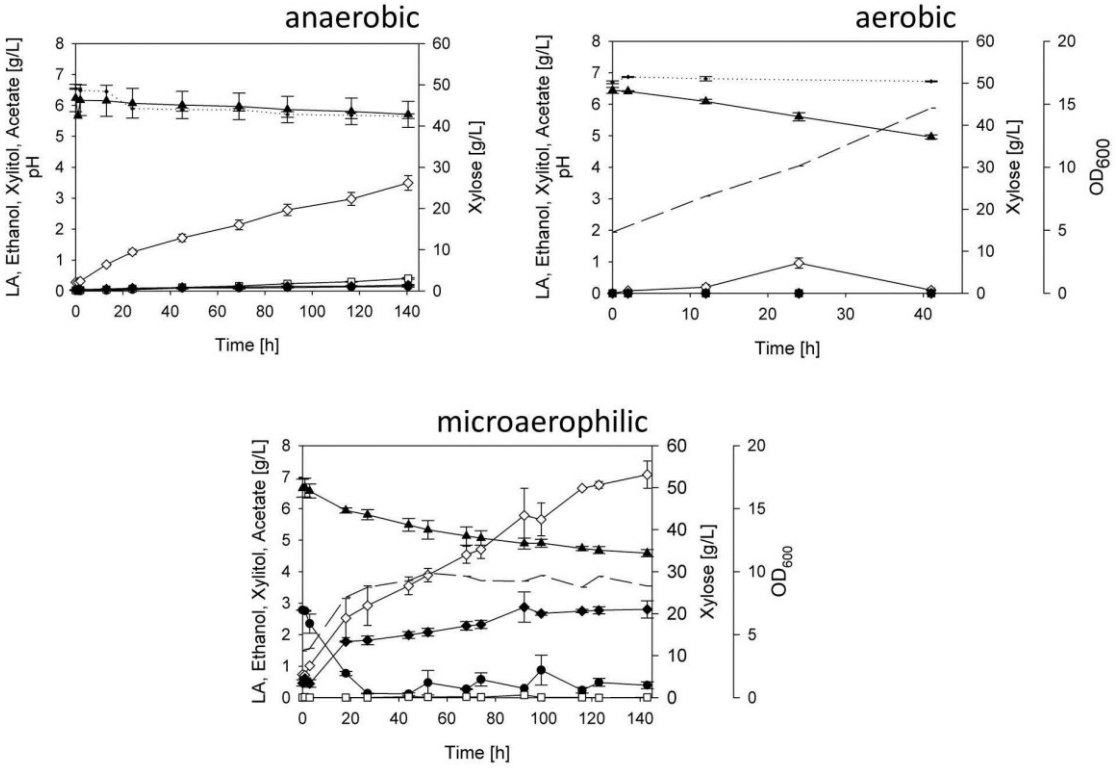

Figure 1. Xylose-to-LA conversions under anaerobic, aerobic and microaerophilic conditions. Data for anaerobic fermentations are taken from [6]. Data points are mean values from two experiments. Symbols: Xylose – filled triangles; lactic acid – empty diamonds; ethanol – filled circles; glycerol – empty triangles; xylitol – empty squares; acetate – filled diamonds; pH – dotted lines; OD_{600} – dashed lines.

Chapter 4

From wheat straw to bioethanol: integrative analysis of a separate hydrolysis and co-fermentation process with implemented enzyme production

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From wheat straw to bioethanol: integrative analysis of a separate hydrolysis and co-fermentation process with implemented enzyme production

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Abstract

Background: Lignocellulosic ethanol has a high potential as renewable energy source. In recent years, much research effort has been spent to optimize parameters involved in the production process. Despite that, there is still a lack of comprehensive studies on process integration. Single parameters and process configurations are, however, heavily interrelated and can affect the overall process efficiency in a multitude of ways. Here, we present an integrative approach for bioethanol production from wheat straw at a representative laboratory scale using a separate hydrolysis and co-fermentation (SHCF) process. The process does not rely on commercial (hemi-) cellulases but includes enzyme production through *Hypocrea jecorina* (formerly *Trichoderma reesei*) on the pre-treated feedstock as key unit operation. Hydrolysis reactions are run with high solid loadings of 15% dry mass pre-treated wheat straw (DM WS), and hydrolyzates are utilized without detoxification for mixed glucose-xylose fermentation with the genetically and evolutionary engineered *Saccharomyces cerevisiae* strain IBB10B05.

Results: Process configurations of unit operations in the benchtop SHCF were varied and evaluated with respect to the overall process ethanol yield ($Y_{Ethanol\text{-}Process}$). The highest $Y_{Ethanol\text{-}Process}$ of 71.2 g ethanol per kg raw material was reached when fungal fermentations were run as batch, and the hydrolysis reaction was done with an enzyme loading of 30 filter paper units (FPU)/$g_{DM\ WS}$. 1.7 ± 0.1 FPU/mL were produced, glucose and xylose were released with a conversion efficiency of 67% and 95%, respectively, and strain IBB10B05 showed an ethanol yield of 0.4 g/$g_{Glc\ +\ Xyl}$ in 15% hydrolyzate fermentations. Based on the detailed process analysis, it was further possible to identify the enzyme yield, the glucose conversion efficiency, and the mass losses between the unit operations as key process parameters, exhibiting a major influence on $Y_{Ethanol\text{-}Process}$.

Conclusions: $Y_{Ethanol\text{-}Process}$ is a measure for the efficiency of the lignocellulose-to-bioethanol process. Based on mass balance analysis, the correlations between single process parameters and $Y_{Ethanol\text{-}Process}$ were elucidated. The optimized laboratory scale SHCF process showed efficiencies similar to pilot scale plants. The herein presented process analysis can serve as effective and simple tool to identify key process parameters, bottlenecks, and future optimization targets.

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Background

Utilization of biomass as renewable and sustainable energy source has called attention from politics and R&D facilities around the world [1-5]. Second-generation bioethanol produced from lignocellulosic waste streams constitutes the most feasible technical option. Reasons are, among many, the geographically evenly distributed and inexpensive feedstock as well as the neat or blended application in the transportation sector without the requirement of major technical adaptation [1-3,6].

The lignocellulose-to-bioethanol process consists of five unit operations; a) pre-treatment of the feedstock, b) production of the (hemi-) cellulolytic enzymes, c) enzymatic hydrolysis of the pre-treated feedstock, d) fermentation of the hydrolyzate to bioethanol, and e) down-stream processing [3,6-10]. In the last two decades, all five unit operations have been subjected to intensive research activities, which resulted in key technologies with improved yields and efficiencies. Thus, different biological, chemical, and physical pre-treatment methods have been developed and are applied alone or in combination to increase the enzyme accessibility and to facilitate cellulose depolymerization [7,9,11]. Further process intensification was achieved by combination of two or more process steps into one single unit operation [5,9], most importantly the simultaneous saccharification and (co-) fermentation (SS(C)F) process which has been applied in bioethanol production at laboratory [12,13] and pilot plant [1,14] scale. Genetic and evolutionary engineering enabled *Saccharomyces cerevisiae* to convert both glucose and xylose, the major hemicellulose-derived sugar, and it enhanced the organism's robustness towards inhibitory compounds (e.g., furans, acids, and phenolic compounds) which are by-products formed during pre-treatment of the feedstock [6,8,10,15-21].

Unit operations within a process are often strongly interlinked so that variation of process parameters in one unit operation can impact the overall process efficiency through indirect effects on other unit operations. Optimization studies, therefore, must implement a complete mass balance-based process analysis and not only focus on single unit operations isolated from the respective others in the process. Despite the extensive research efforts made in R&D facilities in the recent past, there is currently still a lack in comprehensive analyses done at the level of the whole process [1,3]. Besides techno-economic analyses published since the mid-1980s [1,2,4,5,22], data are mainly available from a few pilot scale plants such as the SEKAB plant in Sweden [1,23,24]. The scale of pilot plants, however, already excludes high-throughput analysis of different process configurations. In contrast, data acquired at laboratory scale might not be characteristic and lack transferability for larger scale productions.

In this study, we present an integrative analysis of a lignocellulose-to-bioethanol process on representative laboratory scale (90 mL to 4 L). The process was run as separate hydrolysis and co-fermentation (SHCF), which ensures optimal conditions for both hydrolysis and mixed glucose-xylose fermentation [19,25]. Further, SHCF, as compared to simultaneous saccharification and co-fermentation (SSCF), reduces the complexity of the process, which is important for larger scale applications. Consequently, lignocellulose-to-bioethanol processes which are heading towards industrial scale (e.g., POET-DSM 'Project Liberty,' Clariant AG 'SunLiquid') are run as SHCF. An overview of the three-step process is given in Figure 1. Firstly, a fraction of the pre-treated wheat straw was used for cultivation of a *Trichoderma reesei* strain SVG17 for the production of (hemi-) cellulolytic enzymes. 'On-site' enzyme production is economically advantageous and efficient [26] and it reduces the cost and supply dependency on commercial enzyme suppliers. Despite that, research and pilot scale studies almost exclusively rely on commercially available enzymes [1,2,14,23,26]. The enzyme solution is then utilized for the saccharification of the pre-treated wheat straw (Figure 1). Since fungal cultivation was conducted on the same feedstock, the enzyme mixture is highly optimized for this substrate. The resulting hydrolyzate contains glucose and xylose, and both sugars are converted efficiently to ethanol by the application of the genetically and evolutionary engineered *S. cerevisiae* strain IBB10B05 [19,27].

As shown in Figure 1, the single unit operations were integrated to one SHCF process and mass balance analysis was performed. The resulting overall process ethanol yield ($Y_{\text{Ethanol-Process}}$), the amount of ethanol produced from 1 kg of dry mass feedstock, thereby quantifies the process efficiency, and it is the key parameter to assess and compare different processes [1,2,14,23]. Consequently, mass balance analysis and $Y_{\text{Ethanol-Process}}$ were used in this study to investigate different process configurations, and it generated an in-depth knowledge of the SHCF process.

The aim of this study was to establish a tool for process analysis already on laboratory scale. Integration of unit operations and balance-based analysis of process configurations identified bottlenecks and potential optimization targets within the SHCF process. An integrative process analysis at an early stage can render process development more efficient and therefore might contribute to the success of second-generation bioethanol production.

Results and discussion

The feedstock

The substrate presented in this study was Austrian wheat straw pre-treated with steam explosion. Wheat straw has a high potential as sustainable biomass source in Europe based on its abundance and low cost [28].

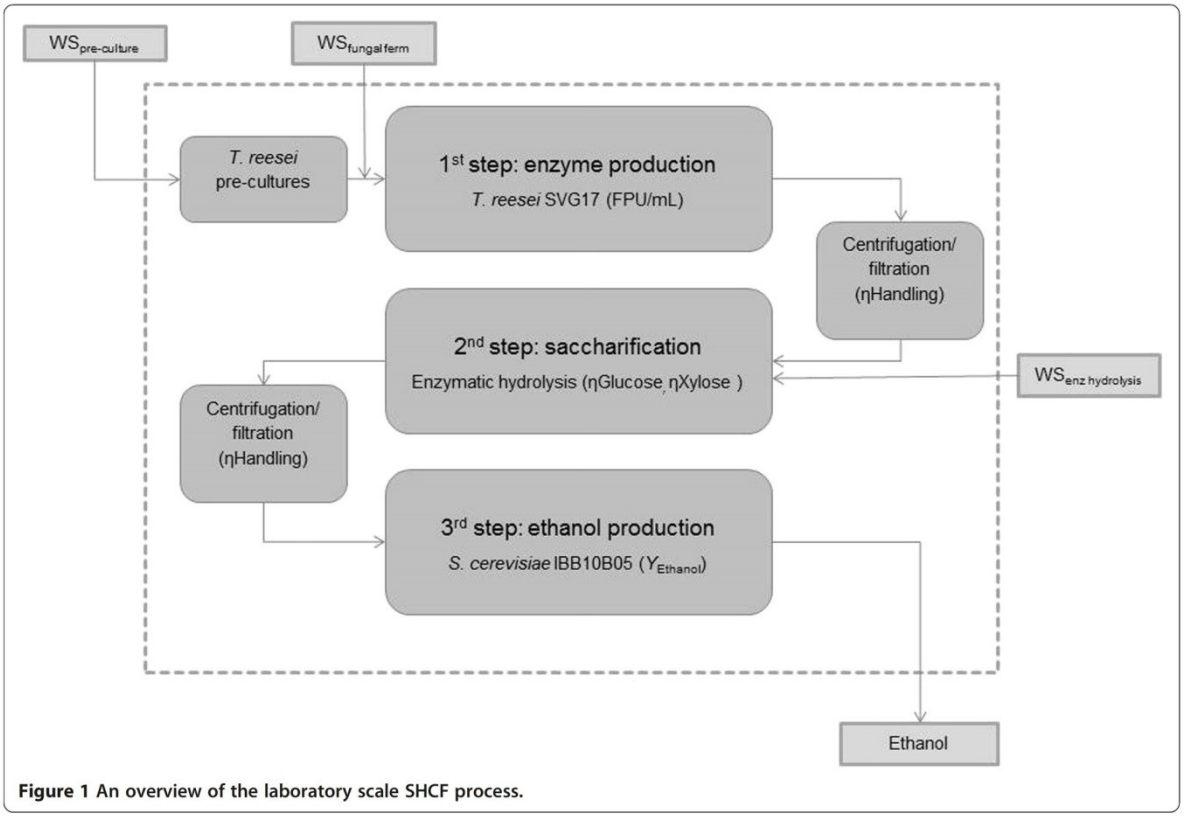

Figure 1 An overview of the laboratory scale SHCF process.

Steam explosion, in combination with chemicals or alone, has been described as an efficient and cost-effective method for pre-treatment of wheat straw [9]. The raw material in this study was treated with a simple method based on steam explosion only. The pre-treated wheat straw had a dry mass (DM) content of approximately 90% and thereof the water insoluble fraction was 69%. The compositional analysis is depicted in Table 1. The acid hydrolyzate contained majorly glucose and xylose. Other hemicellulose-derived sugars (e.g., L-arabinose, galactose) were only present in amounts below the detection limit of the high performance liquid chromatography (HPLC) system and are not mentioned in Table 1. Although processed under the same conditions, the pre-treated wheat straw composition showed batch-to-batch variability. Thus, the glucose and xylose content varied by 5% and 13%, respectively (Table 1). Variation in the pre-treated feedstock composition, especially in xylose content, has been observed before [25]. The amorphous nature of the hemicellulose and different levels of degradation during the pre-treatment as well as seasonal variations are likely explanations. Since both batches of wheat straw were utilized throughout this study, analysis of unit operation and mass balanced process analysis were based on averaged values (Table 1). The raw material before pre-treatment had a xylose content of 24.9 ± 0.4% and a glucose content of 36.1 ± 0.1% dry matter (data not shown). Mass losses caused by pre-treatment were 10% on average.

Table 1 Compositional analysis of the pre-treated wheat straw

Components in dry matter	Percentage [%]		
	Batch 1	Batch 2	Mean
Carbohydrates			
Glucose	43.7	48.7	46.2 ± 2.5
Xylose	17.2	13.1	15.2 ± 2.0
Non-carbohydrates			
Acid-soluble lignin	1.3	2.0	1.7 ± 0.3
Acid-insoluble lignin	27.7	30.0	28.9 ± 1.2
Ashes	4.5	4.4	4.5 ± 0.0
Others	5.6	4.3	4.9 ± 0.6

Experimental analysis of unit operations

The three unit operations of the SHCF process, enzyme production, hydrolysis, and fermentation, were analyzed under varying process conditions. As shown in Figure 1, the process streams between the unit operations were

treated by centrifugation, filtration, and concentration. The resulting losses were included into the process analysis with the efficiency factor of conditioning steps (ηHandling), and it was determined to be 75% on average.

Production of (hemi-) cellulases by T. reesei SVG17

T. reesei is the majorly applied organism for (hemi-) cellulase production, and it has been studied and continuously improved since the 1960s [29-33]. The herein presented *T. reesei* SVG17 is a mutant of the QM9414 strain, and previous studies have described it as useful enzyme producer at both laboratory and pilot scale [31]. Fungal cultivations were run as batch fermentation with a substrate loading of 30 g DM pre-treated wheat straw per L ($g_{DM\ WS}$/L). In 7 days of fermentation, a total volumetric cellulolytic activity of 1.7 ± 0.1 FPU/mL was reached. The beta-glucosidase activity was determined to be 0.6 ± 0.1 U/mL. To increase the enzyme yields, fermentations were also run as fed-batch. Per 2 L of fermentation, 30 $g_{DM\ WS}$ was added three times after 66, 94, and 138 h of fermentation. The time course of the fed-batch fermentation is depicted in Figure 2A. In 210 h of fermentation, the volumetric cellulase activity reached 2.7 ± 0.02 FPU/mL. Similar to the batch fermentation, the beta-glucosidase activity was approximately half of the FPU/mL value and it was determined to be 1.5 ± 0.02 U/mL. Addition of feedstock was described to prolong the phase in which enzyme production is most active by freshly inducing both biomass growth and enzyme expression [30,32]. Consequently, the cellulase activity is increasing after each addition of WS, and only towards the end (180 to 210 h), the FPU/mL-time curve is stagnating. The time courses of beta-glucosidase activity and protein concentration show a similar pattern with a less pronounced effect of the feed. In the fed-batch fermentation, it was possible to improve the volumetric cellulase activity 1.6-fold as compared to the batch fermentation. This increase, however, required a 2.5-fold higher substrate loading. The impact of both process configurations on $Y_{Ethanol\text{-}Process}$ was evaluated with mass balance analysis and will be discussed hereinafter.

Despite the difficult substrate conditions, *T. reesei* SVG17 was able to grow on the pre-treated wheat straw and showed efficient and reproducible production of (hemi-) cellulolytic enzymes. A drawback of strain SVG17 is the relatively low beta-glucosidase activity in the enzyme solution which did not exceed 50% of the overall cellulase activity (measured in FPU). A beta-glucosidase activity to FPU ratio of 1 was found to be the lower limit for efficient biomass conversion [34,35]. Low beta-glucosidase activity is an often observed problem in the production of cellulolytic enzymes by *T. reesei*. To overcome this problem, genetically engineered strains overexpressing heterologous beta-glucosidase genes have been described in the literature [34-37]. Although the availability of a robust *T. reesei* strain producing a beta-glucosidase-boosted enzyme solution could be important in view of an optimized process output, the aim of this study was not primarily optimization itself, but rather provision of a *basis for optimization* through integrative mass balance analysis of a representative SHCF process. Therefore, the use of *T. reesei* strain SVG17 was fully in line with the concept of the study, and the limitation in beta-glucosidase noted was not considered to restrict the relevance of the current investigation. Moreover,

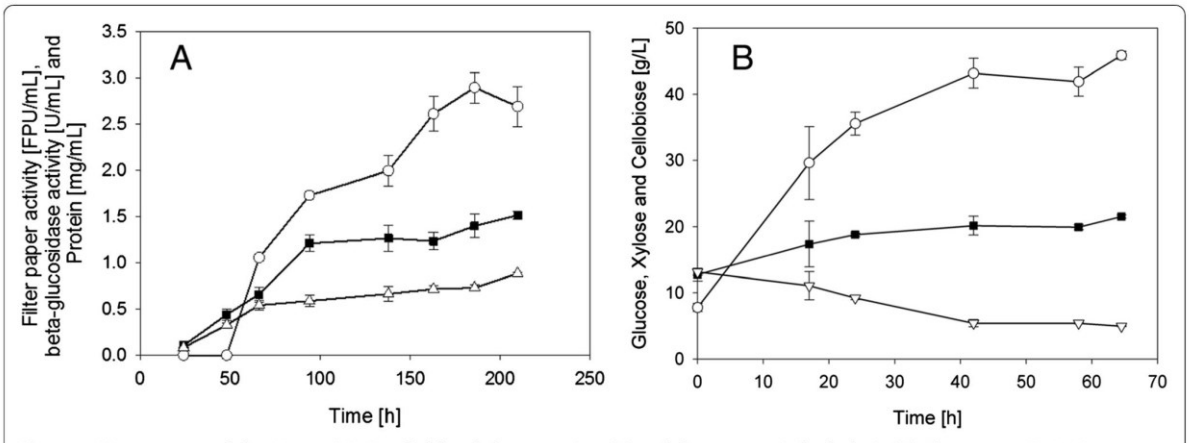

Figure 2 Time courses of the *T. reesei* SVG17 fed-batch fermentation (A) and the enzymatic hydrolysis (B). Thirty grams DM WS was added after 66, 94, and 138 h to the fungal fermentation. Hydrolysis reaction was run with 30 FPU/$g_{DM\ WS}$. Both time courses represent mean values from two experiments. Symbols: **(A)** Total cellulase activity (empty circles), beta-glucosidase activity (filled squares), and protein concentration (empty triangles). **(B)** Glucose (empty circles), xylose (filled squares), and cellobiose (empty triangles).

the effects of enhanced beta-glucosidase activity on hydrolysis yield and process performance are described later in this manuscript.

Enzymatic hydrolysis

To render lignocellulose-to-bioethanol processes economically feasible, a high ethanol titer is crucial [17]. Consequently, hydrolysis reactions must aim for high solid loadings to increase the sugar content. This, in turn, does increase the content of compounds, which are potentially toxic for the fermentation organism. In a previous study, we have shown that the solid loading in the hydrolysis reaction can be increased from 5% to 15% DM WS without introducing severe inhibition effects on *S. cerevisiae* strain IBB10B05 [19]. Enzyme loadings were varied. Firstly, reactions were run with 25 FPU/$g_{DM\ WS}$ [19,27] and the final hydrolyzate contained 40.6 ± 5.7 g/L glucose and 18.0 ± 2.3 g/L xylose. Based on the compositional analysis of the feedstock (Table 1), this equals a conversion efficiency of 60% for glucose (ηGlucose) and 81% for xylose (ηXylose). To improve the conversion efficiencies, the enzyme loading was increased to 30 FPU/$g_{DM\ WS}$, and ηGlucose of 67% and ηXylose of 95% were reached. The 15% hydrolyzate contained 46.1 ± 0.2 g/L glucose and 21.5 ± 0.2 g/L xylose. The time course of the 30 FPU/$g_{DM\ WS}$ hydrolysis reaction is depicted in Figure 2B. In addition to glucose and xylose, a considerable amount of cellobiose (5.0 g/L, Figure 2) was released into the hydrolyzate. Cellobiose is known to have an inhibitory impact on cellulases (e.g., [38,39]). Accumulation of cellobiose during hydrolysis is caused by limitation in beta-glucosidase activity in the enzyme mixture used. To evaluate the impact of enhanced beta-glucosidase activity, hydrolysis reactions (30 FPU/$g_{DM\ WS}$) were additionally performed with supplemented Novozyme188. The FPU to beta-glucosidase activity was 1, which was chosen according to the literature [34,35]. The resulting hydrolyzate had a glucose and xylose concentration of 51.8 ± 1.1 g/L and 23.0 ± 1.0 g/L, respectively. The cellobiose concentration was below 1.5 g/L. Addition of beta-glucosidase increased the ηGlucose from 67% to 78%. The already high ηXylose was further increased and reached full conversion.

Fermentation to ethanol with S. cerevisiae strain IBB10B05

S. cerevisiae IBB10B05 proved to be a sturdy and efficient fermentation strain for mixed glucose-xylose fermentation in spent sulfite liquor, wheat straw hydrolyzates, and a combination thereof [19,27]. Strain IBB10B05 performs excellently under most basic process and substrate conditions. Thus, fermentations were run in simple batch cultures without process monitoring and control (e.g., pH adjustment). The 15% hydrolyzate was applied without pre-treatment (e.g., detoxification) and yeast extract was added as a sole substrate supplement. An in-depth physiological characterization of strain IBB10B05 in fermentation of 15% hydrolyzates has been published recently [19]. The fermentation time course is depicted in Additional file 1. Both glucose (~37 g/L) and xylose (~19 g/L) were depleted within 50 h of fermentation, and in total, 22 g/L ethanol was produced within this time frame (Additional file 1, [19]). This represents an ethanol yield of ~0.4 $g/g_{Glc\ +\ Xyl}$.

Integration of unit operations and process analysis

The single unit operations, enzyme production, hydrolysis, and fermentation, were integrated to one SHCF process as depicted in Figure 1. To evaluate and compare the different process configurations in context of the complete SHCF process, mass balance analysis was performed. The different process configurations are described based on critical output parameters (FPU/mL, ηGlucose, ηXylose, ηHandling, and ethanol yield ($Y_{Ethanol}$)), and the corresponding mass balance analyses are summarized in Additional file 2. A comparison of process configurations based on $Y_{Ethanol\text{-}Process}$ is depicted in Figure 3. Note that to account for losses caused by pre-treatment and to facilitate comparison of the laboratory scale SHCF process with data from the literature, mass balance analyses were based on the raw material. It was assumed that the required input of raw material was 10% higher as the input calculated for the pre-treated wheat straw (Additional file 2). Throughout this study, $Y_{Ethanol\text{-}Process}$ is given in g ethanol produced per kg raw material ($g_{Ethanol}/kg_{DM\ RM}$).

Enzyme production, the first unit operation of the SHCF process, was run as batch or as fed-batch. The latter approach resulted in a higher volumetric activity but also required a higher substrate loading ('Production of (hemi-) cellulases by *T. reesei* SVG17'). When enzymes were produced in batch fermentations (Config.1), the resulting $Y_{Ethanol\text{-}Process}$ was 71.2 $g_{Ethanol}/kg_{DM\ RM}$. In fed-batch fermentations (Config.2), $Y_{Ethanol\text{-}Process}$ was 58.0 $g_{Ethanol}/kg_{DM\ RM}$, which is a 19% decrease as compared to Config.1. This clearly emphasizes the need for evaluating process parameters in context of the complete process. Despite the extensive research on cellulase production in *T. reesei* (e.g., [29,32,40,41]), the influence of substrate or process conditions on the success of the overall bioethanol production process is scarcely considered. Enzymatic hydrolysis, the second unit operation, was analyzed with two different enzyme loadings, 25 FPU/$g_{DM\ WS}$ (Config.3) and 30 FPU/$g_{DM\ WS}$ (Config.1). Config.3 resulted in an overall process ethanol yield of 67.5 $g_{Ethanol}/kg_{DM\ RM}$ (Additional file 2, Figure 3). This is 5% less as compared to the reaction with 30 FPU/$g_{DM\ WS}$ ($Y_{Ethanol\text{-}Process}$ 71.2 $g_{Ethanol}/kg_{DM\ RM}$). The last unit operation, the fermentation to ethanol, was accomplished with the

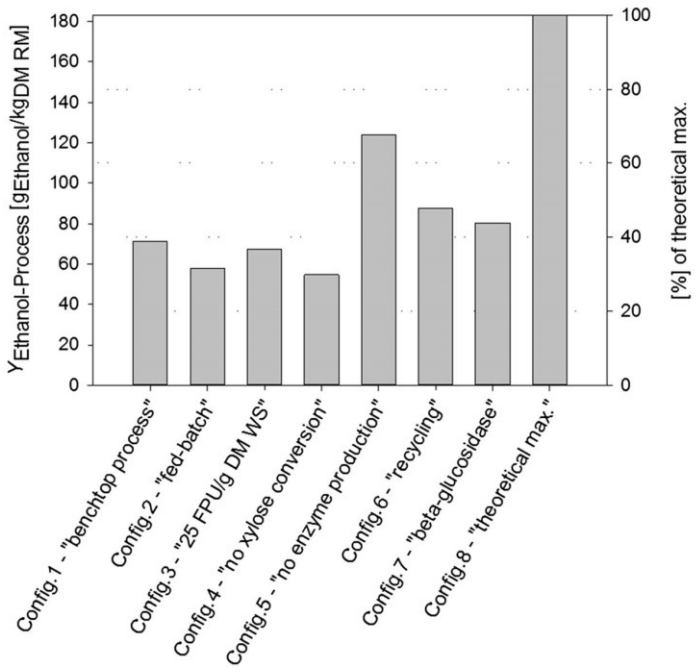

Figure 3 The influence of different process configurations on $Y_{Ethanol\text{-}Process}$. Detailed description of process parameters are summarized in Additional file 2.

xylose-fermenting *S. cerevisiae* strain IBB10B05 (Figure 1). To compare the efficiencies of a SHCF and a separate hydrolysis and fermentation (SHF) process, mass balance analysis was additionally performed with glucose conversion only (Config.4). With an ethanol on glucose yield of 0.45 g/g_{Glc} [19,27], the $Y_{Ethanol\text{-}Process}$ was 54.6 $g_{Ethanol}/kg_{DM\ RM}$ (Additional file 2, Figure 3). This is 23% less as compared to Config.1. Although, genetically engineered xylose-fermenting *S. cerevisiae* strains have been described extensively in the literature (e.g., [10,15]), pilot plants often still operate with non-GM yeasts and rely on glucose conversion only [1,23]. This integrative process analysis, however, clearly highlights the importance of efficient conversion of all sugars in the hydrolyzates and strain IBB10B05 proved to be an excellent candidate.

Integration of unit operations and analyses of the different process configurations (Config.1 to 3) showed that Config.1 has the highest $Y_{Ethanol\text{-}Process}$ (Figure 3) and the mass balance analysis is depicted in Table 2. Thus, enzyme production in batch fermentation (working volume 4 L) and processing of the enzyme solution (ηHandling 75%) resulted in a total of 5,100 FPU. With an enzyme loading of 30 $FPU/g_{DM\ WS}$, it was possible to hydrolyze 170 g DM WS. After treatment of the 15% hydrolyzate (ηHandling 75%), 39.5 g glucose and 18.4 g xylose were available for fermentation, which was converted to 23.1 g of ethanol. A total process ethanol yield of 71.2 $g_{Ethanol}/kg_{DM\ RM}$ was reached (Table 2).

To assess the efficiency, the benchtop SHCF process was compared to currently available data from pilot (SEKAB, IBUS, BCyL) and commercial (Clariant, 'SunLiquid') scale plants. A summary of process configurations, plant capacities, and $Y_{Ethanol\text{-}Process}$ is depicted in Table 3. The pilot scale plants summarized in Table 3 operate without 'on-site' production of (hemi-) cellulolytic enzymes. To still render a comparison of the process efficiencies possible, mass balance analysis of the laboratory scale SHCF process was performed excluding enzyme production (Config.5, Figure 3 and Additional file 2). Without the loss of feedstock required for the fungal cultivation, the $Y_{Ethanol\text{-}Process}$ was 123.7 $g_{Ethanol}/kg_{DM\ RM}$, which is 1.7-fold higher as compared to Config.1. This is already within the range of $Y_{Ethanol\text{-}Process}$ reported for the pilot scale plants (118 to 157.8 3 $g_{Ethanol}/kg_{DM\ RM}$; Table 3), clearly highlighting the usefulness of the herein presented laboratory scale SHCF process as a model for establishing an integrative process analysis. However, direct comparison of Config.1 and Config. 5, solely based on $Y_{Ethanol\text{-}Process}$, is not sufficient. 'On-site' enzyme production has been described to entail several advantages, such as being cost-effective and

Table 2 Mass balance analysis of the benchtop SHCF process

	Input	Output
1st step: enzyme production (*T. reesei* SVG17)		
Pre-cultures ($WS_{pre\text{-}culture}$)	5.6 g DM WS	
Batch cultivation ($WS_{fungal\ ferm}$)	120 g DM WS	
Total cellulolytic activity		1.7 FPU/mL
ηHandling 75%		5,100 FPU total
2nd step: saccharification (enzymatic hydrolysis)		
Substrate loading ($WS_{enz\ hydrolysis}$)	170 g DM WS	
Glucose (ηGlucose 67%, ηHandling 75%)		39.5 g
Xylose (ηXylose 95%, ηHandling 75%)		18.4 g
3rd step: ethanol production (*S. cerevisiae* IBB10B05)		
Ethanol ($Y_{Ethanol}$ 0.4 $g/g_{Glc+Xyl}$)		23.1 g
Total		
Substrate loading - pre-treated	295.6 g DM WS	
Substrate loading - raw material	325.2 g DM RM	
Ethanol		23.1 g
$Y_{Ethanol\text{-}Process}$	71.2 $g_{Ethanol}/kg_{DM\ RM}$	

An overview of the process is depicted in Figure 1. Boundary conditions: batch fermentations with 30 $g_{DM\ WS}$/L, 15% DM WS, and 30 FPU/$g_{DM\ WS}$.

efficient [26,42]. Thus, for further evaluation of the feasibility of the process configurations, a detailed economic analysis, as published for other process [2,14,22], must be conducted.

The by far most efficient process is the 'SunLiquid' process, which is operated as SHCF with implemented 'on-site' enzyme production. The reported $Y_{Ethanol\text{-}Process}$ is 222.2 $g_{Ethanol}/kg_{DM\ RM}$ (Table 3) [42]. This is 18% higher as compared to the maximum theoretical yield of the benchtop SHCF process which was determined to be 183.1 $g_{Ethanol}/kg_{DM\ RM}$ (Config.8, Figure 3 and Additional file 2). There are several factors that could explain the exceptionally high $Y_{Ethanol\text{-}Process}$ described for the 'SunLiquid' process. The first factor is the pre-treatment method applied. Ideally, pre-treatment of lignocellulosic biomass enriches the structural carbohydrate cellulose and hemicellulose by removal of the lignin and enhances the accessibility of the partially crystalline cellulose. It therefore has an impact on the efficiency of the fungal fermentation and the enzymatic hydrolysis and is directly affecting $Y_{Ethanol\text{-}Process}$ [7,11]. In this study, the steam explosion was performed as batch and running the process continuously could reduce mass losses caused by pre-treatment. However, evaluation of varying pre-treatment methods was beyond the scope of this study and is not considered in more detail hereinafter. The second factor influencing $Y_{Ethanol\text{-}Process}$ is the potential application of enzyme or solid recycling to boost the hydrolysis efficiency [43,44]. This process option was also analyzed in context of this study. With an estimated increase in glucose released per enzyme loading of 35% (e.g., [43,44]), the $Y_{Ethanol\text{-}Process}$ of the benchtop SHCF process would improve to 87.8 $g_{Ethanol}/kg_{DM\ RM}$ (Config.6, Figure 3 and Additional file 2). The third factor is the organism employed for enzyme production. The importance of a *T. reesei* strain secreting a balanced enzyme mixture has been described in the literature [34-37]. In this study, the application of an enzyme solution with a beta-glucosidase to FPU ratio of 1 was investigated. The resulting $Y_{Ethanol\text{-}Process}$ was 80.3 $g_{Ethanol}/kg_{DM\ RM}$ (Config.7, Figure 3 and Additional file 2). In comparison to Config.1, the altered process configurations resulted in

Table 3 $Y_{Ethanol\text{-}Process}$ for commercial, pilot, and laboratory scale processes

Substrate	Capacity [1,000 $t_{DM\ RM}$/year]	Process	$Y_{Ethanol\text{-}Process}$ [$kg_{Ethanol}/t_{DM\ RM}$]	Reference
Cereal straw	150	SHCF + E[a]	222.2	Clariant 'SunLiquid' [42]
Wheat straw	8.8	SSF	123	'IBUS' [23]
Forestry residues	0.7	SSF	118 to 157.8	'SEKAB' [1]
Wheat straw	25.6	SHF	154	'BCyL' [1]
Wheat straw	-	SHCF/+E[a]	123.7/71.2	This study

[a]'On-site' enzyme production.

an increase in $Y_{\text{Ethanol-Process}}$ of 19% (Config.6) and 11% (Config.7). However, the process yields did not exceed 50% of the reported $Y_{\text{Ethanol-Process}}$ of the 'SunLiquid' process. Therefore, we took the process analysis one step further and analyzed the benchtop SHCF process towards potential bottlenecks.

Based on the detailed mass balance analysis of the integrated benchtop SHCF process and the resulting understanding of the process streams, it was possible to identify three key parameters which exhibit a significant influence on $Y_{\text{Ethanol-Process}}$, the enzyme yield, ηGlucose, and ηHandling (Figure 1). The correlations between $Y_{\text{Ethanol-Process}}$ and these parameters are depicted in Figure 4. Firstly, $Y_{\text{Ethanol-Process}}$ was plotted over the enzyme yield (FPU/mL) (Figure 4A). To increase the $Y_{\text{Ethanol-Process}}$ of Config.1 to 100 $g_{\text{Ethanol}}/kg_{\text{DM RM}}$, for an instance, a 3.1-fold increase in total cellulolytic activity is required (from 1.7 to 5.3 FPU/mL, Figure 4A). The importance of an efficient enzyme production is further highlighted, by comparing the maximum theoretic $Y_{\text{Ethanol-Process}}$ observed in this study with the 'SunLiquid' process. To enhance the $Y_{\text{Ethanol-Process}}$ of Config.8 to 222 $g_{\text{Ethanol}}/kg_{\text{DM RM}}$ [42], the enzyme yield must be increased twofold, from 1.7 FPU/mL to 3.4 FPU/mL. In addition to the above discussed factors, a higher enzyme yield during fungal fermentation might therefore explain for the high $Y_{\text{Ethanol-Process}}$ of the 'SunLiquid' process.

As shown in Figure 4B, the overall process efficiency is further influenced by the two parameters ηGlucose and ηHandling. Thus, $Y_{\text{Ethanol-Process}}$ can vary between 22.4 (ηGlucose 25% and ηHandling 50%) and 127.2 $g_{\text{Ethanol}}/kg_{\text{DM RM}}$ (ηGlucose 95% and ηHandling 95%) under else same boundary conditions. Possible improvement of ηGlucose has already been described within this study. Data summarized in Table 3 suggest that $Y_{\text{Ethanol-Process}}$ increases with the scale of the plant ('Capacity'). A correlation between plant capacity and overall yield is further supported by the literature [1-5,14,22-24], and it is suggested that processes are becoming more efficient with increasing scale [1-3,5]. Explicit information on ηHandling for pilot and commercial scale plants are not given. The fact that $Y_{\text{Ethanol-Process}}$ of Config.5 ("no enzyme production") is within the range of to the pilot scale plants (Table 3), however, might indicate that the ηHandling are similar. To further increase $Y_{\text{Ethanol-Process}}$, improvement of ηHandling will be a target of future optimization studies of the benchtop SHCF process.

Conclusions

In this study, an integrative process analysis of a benchtop SHCF is presented. Based on mass balance analysis, the influence of varying process configurations on $Y_{\text{Ethanol-Process}}$ was analyzed. Thereby, a fundamental understanding of the complete process was established. This allowed for identification of the process parameters, which have the highest impact on $Y_{\text{Ethanol-Process}}$, the enzyme yield, ηGlucose, and ηHandling. It was further shown that, under comparable process conditions (Conifg.5 - no enzyme production), $Y_{\text{Ethanol-Process}}$ of the benchtop SHCF process is equal to pilot scale plants. We therefore believe that the benchtop-scale analysis described herein presents an important and useful tool to identify bottlenecks and optimization targets within the process with reasonable effort and expenditure.

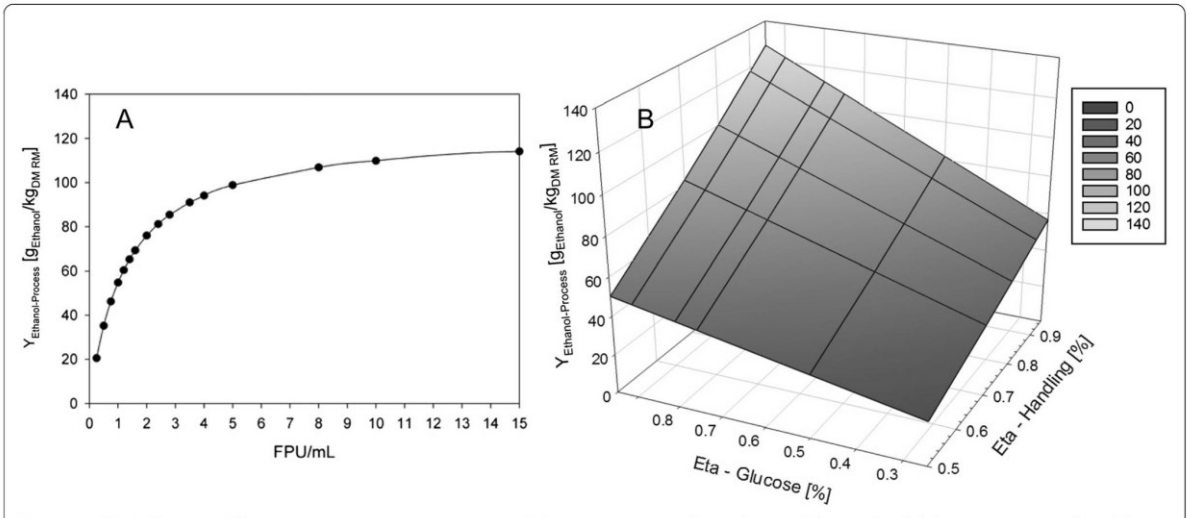

Figure 4 The influence of key parameters on $Y_{\text{Ethanol-Process}}$. (A) $Y_{\text{Ethanol-Process}}$ in dependence of the total cellulolytic activities produced by fungal fermentation (boundary conditions: batch with 30 $g_{\text{DM WS}}$/L, 30 FPU/$g_{\text{DM WS}}$, ηGlucose 67%, ηXylose 95%, ηHandling 75%, and Y_{Ethanol} 0.4 g/$g_{\text{Glc+Xyl}}$). **(B)** Influence of ηGlucose and ηHandling on $Y_{\text{Ethanol-Process}}$ (boundary conditions: batch with 30 $g_{\text{DM WS}}$/L, 1.7 FPU/mL, 30 FPU/$g_{\text{DM WS}}$, ηXylose 95%, and Y_{Ethanol} 0.4 g/$g_{\text{Glc+Xyl}}$).

Findings of this study stress the importance to establish a mass balance-based understanding of the lignocellulose-to-bioethanol process and to optimize unit operations or process parameters with respect to $Y_{\text{Ethanol-Process}}$.

Methods

Chemicals and media used

Unless mentioned differently, all chemicals were from Carl Roth + Co KG (Karlsruhe, Germany). Mineral (M-) media for cultivation of *T. reesei* SVG17 contained 5 g/L yeast extract, 5 g/L KH_2PO_4, 3.75 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4 \times 7H_2O$, 0.3 g/L $CaCl_2 \times 2H_2O$, and 1 mL/L trace elements (5 g/L $FeSO_4 \times 7H_2O$, 1.6 g/L $MnSO_4 \times H_2O$, 1.4 g/L $ZnSO_4 \times 7H_2O$, 0.2 g/L $CoCl_2 \times 6H_2O$, 15 g/L EDTA disodium chloride salt). Additionally, 0.2 mL/L Tween 80 (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 g/L rapeseed oil were supplied. Potato-dextrose-agar (PDA) was prepared as described by the manufacturer. Yeast extract peptone glucose (YPD) medium for cultivation of *S. cerevisiae* contained 10 g/L yeast extract, 20 g/L peptone from casein, and 20 g/L glucose. In 15% hydrolyzate conversion experiments, 10 g/L of yeast extract was added as a sole medium supplement.

Preparation of the lignocellulosic feedstock

Pre-treated wheat straw from Austria was kindly provided by the University of Applied Sciences in Upper Austria (FH Wels). Throughout this study, two batches of feedstock were received and they were both pre-treated as described in the following. The wheat straw was air-dried to a water content of approximately 8% (*w*/*w*), and the fibers were chaffed to reduce the length of the fibers to below 8 cm. Further, the wheat straw was treated by steam explosion at 200°C, 15 bar for 10 min, with a water to wheat straw ratio of 1. After cooling, the wheat straw was dried to a dry mass content of 90% and stored at 4°C in plastic bags. Per batch, DM and water insoluble (WIS) content was analyzed in triplets. For the former, a moisture analyzer (MA 50, Sartorius AG, Göttingen, Germany) operated at 105°C was used. For determination of the WIS content, 2 g of wheat straw was washed with 50 mL of 50°C warm water, dried at 105°C for 24 h, and weighted. The content of structural carbohydrates, lignin, and ash in the wheat straw was analyzed following the protocol of the National Renewable Energy Laboratory (NREL) [45].

Strains

For the production of the (hemi-) cellulolytic enzymes, the *T. reesei* strain SVG17 was applied. The strain background has been described in a previously published study [31]. For conversion of the 15% hydrolyzate to ethanol, the *S. cerevisiae* strain IBB10B05 was utilized. It is a descendant of the BP10001 strain, which was further improved by evolutionary engineering. Detailed descriptions of the construction of the BP10001 strain and the evolution strategy have been published elsewhere [18,20].

Bioreactor cultivations for the production of the (hemi-) cellulolytic enzymes

T. reesei SVG17 was maintained on PDA plates at 4°C. The fungus was revitalized by transfer of a piece of overgrown agar onto fresh PDA plates. Incubation was for 3 days at 30°C. Starter cultures were prepared in 300-mL wide mouth shake flasks without baffles filled with 200 mL of mineral medium (M-medium) containing 14 $g_{DM\ WS}$/L. Fermentation was started by transfer of a piece of overgrown PDA agar to the fermentation media. Incubation was for 5 days at 30°C, 200 rpm, and pH 4.5 in a Certomat BS-1 orbital incubator shaker (Sartorius AG, Göttingen, Germany). Subsequently pre-cultures were pooled to ensure homogeneous biomass composition.

Main cultivations were run in batch or fed-batch mode in benchtop bioreactors (Labfors III, Infors AG, Bottmingen, Switzerland) with 2 L as working volume. The bioreactors are equipped with two six-plated impellers. The reactor diameter to impeller diameter ratio was 3 and the reactor height to reactor diameter ratio was 1.5. Cultivations were accomplished in duplicates (two parallel bioreactors). M-media was supplemented with 30 $g_{DM\ WS}$/L, and the fermentation was started by transfer of 200 mL of the pooled pre-cultures into the bioreactor. Fermentation conditions were as follows: 30°C, pH 4.5 (adjusted online with 1 M KOH and 3%, by volume NH_3), and a dissolved oxygen concentration of 20%. The latter was adjusted continuously with an agitation (200 to 800 rpm) and aeration (0.16 to 1.5 L/min pressurized air) cascade. Incubation was for 7 days. In fed-batch mode, 30 g DM WS and media supplements for 1 L of fermentation media was added per bioreactor after 66, 94, and 138 h of fermentation. Fed-batch experiments were prolonged to 9 days of fermentation time. During the fermentation, samples were frequently removed and centrifuged (15,700*g*, 4°C for 10 min, Eppendorf 5415 R, Eppendorf, Hamburg, Germany) and the supernatant stored at 4°C for analysis. Enzyme activity was evaluated in beta-glucosidase and total cellulolytic activity (given in filter paper units, FPU/mL). FPU activity was measured following International Union of Pure and Applied Chemistry (IUPAC) recommendations [46]. Beta-glucosidase activity was determined as described previously [47], with the following alterations. Two hundred fifty microliters of diluted enzyme solution was mixed with 250 μL of 2.0 mM para-nitrophenyl-β-D-glucopyranoside solution (in 50 mM sodium acetate buffer, pH 5.0) and incubated at 50°C for 10 min. The reaction was stopped with 1 M Na_2CO_3. Protein concentration was determined with the Bradford method [48]. Proteins were

precipitated and quantified utilizing the Roti-Quant kit (Roth) and following the manufacturer's instructions.

The enzyme solution was harvested and prepared for the subsequent enzymatic hydrolysis as described in the following. The supernatant of the fungal cultivation was collected by centrifugation (4,420*g*, 4°C; 20 min; Sorvall RC-5B; Thermo Fisher Scientific Inc., Waltham, MA, USA), concentrated by evaporation (45°C, 40 mbar, Laborota 4000, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) to one tenth of its original volume, filtrated sterile (Whatman Klari-Flex System; GE Healthcare, Little Chalfont, UK), and stored at 4°C. The loss of enzyme solution was included into the mass balance analysis with the efficiency factor ηHandling (Figure 1) and was determined by measuring volume and mass before and after the processing steps.

Enzymatic hydrolysis of the wheat straw

The substrate loading for the hydrolysis reaction was 15%, by weight dry mass wheat straw. Two different enzyme loadings were applied, 25 and 30 FPU/$g_{DM\ WS}$. Additionally, one hydrolysis reaction (30 FPU/$g_{DM\ WS}$) was performed with supplemented beta-glucosidase (Novozyme188; Sigma-Aldrich) to a total activity of 30 U/$g_{DM\ WS}$. Reactions were performed in 10 mM sodium acetate buffer (pH 4.8) in 500-mL shake flaks with ground-in glass stoppers. Total mass of the reaction was 260 g. The wheat straw suspension was autoclaved and the enzyme added aseptically. Incubation was 50°C, 200 rpm for 72 h (Certomat BS-1). During the hydrolysis, samples were taken. Immediate sample work-up included the boiling of the reaction mixture (100°C, 15 min. 300 rpm, Thermomixer 'comfort' Eppendorf) and centrifugation and storage of the supernatant at −20°C for HPLC analysis.

The hydrolyzate was prepared for the subsequent conversion to bioethanol as described in the following. Firstly, it was heated to 100°C for 15 min in a water bath. Remaining solids were removed by centrifugation (4,420*g*, 4°C, 10 min, Sorvall RC-5B) and discarded. The pH of the supernatant was adjusted to 6.5 with 1 M NaOH, and it was filtrated sterile ('Klari-Flex') and stored at 4°C. Mass and volume loss was determined before and after the processing of the hydrolyzate and included into the mass balance analysis as ηHandling.

Shaken-bottle cultivation for bioethanol fermentation

Fermentation of 15% hydrolyzate to ethanol utilizing the *S. cerevisiae* strain IBB10B05 was accomplished as described previously [19]. In brief, seed and starter cultures were prepared aerobically in shaken flask cultures in YPD media at 30°C. Main cultivations were performed anaerobically at 30°C in glass bottles (working volume 90 mL) tightly sealed with rubber septa. Eighty percent of the total volume was wheat straw hydrolyzate, and the remainder volume was composed of 10% yeast extract and 10% inoculum. The starting optical density at 600 nm (OD_{600}) was 5. Incubation was at 30°C and 180 rpm (Certomat BS-1) for 7 days. During the fermentation, samples (1.5 mL) were frequently removed from the fermentation media and centrifuged (10 min, 15,700*g*, 4°C, Eppendorf 5415R) and the supernatant stored at −20°C for HPLC analysis.

HPLC analysis of sugars and metabolites

Sugars (glucose, xylose, arabinose, galactose, and cellobiose) as well as extracellular metabolites (ethanol, glycerol, xylitol, and acetate) were analyzed by HPLC (Merck-Hitachi LaChrome system equipped with an L-7250 autosampler, a Merck L-7490 RI detector, and a Merck L-7400 UV detector). The system was equipped with an Aminex HPX-87H column and an Aminex Cation H guard column (both Bio-Rad Laboratories, Hercules, CA, USA). The operation temperature was 65°C for both columns and the flow rate of the mobile phase (5 mM sulfuric acid) was 0.6 mL/min. The hemicellulose-derived sugars (galactose and arabinose) were only present in minor amounts (<0.5 g/L) and are therefore not further mentioned.

Additional files

Additional file 1: Mixed glucose-xylose fermentation in 15% hydrolyzate utilizing *S. cerevisiae* strain IBB10B05. Time course was derived from a previous publication [19]. Symbols: glucose (crosses), xylose (full diamonds), glycerol (empty triangles), xylitol (empty squares), ethanol (empty circles).

Additional file 2: Summary of in- and output parameters and mass balance analyses of the different process configurations.

Abbreviations

SHF: separate hydrolysis and fermentation; SSF: simultaneous saccharification and fermentation; SHCF: separate hydrolysis and co-fermentation; SSCF: simultaneous saccharification and co-fermentation; DM: dry mass; WS: pre-treated wheat straw; RM: raw material; FPU: filter paper unit; Glc: glucose; Xyl: xylose; HPLC: high performance liquid chromatography; IUPAC: International Union of Pure and Applied Chemistry; M-medium: mineral medium; NREL: National Renewable Energy Laboratory; OD_{600}: optical density at 600 nm; WIS: water-insoluble; $Y_{Ethanol}$: ethanol yield; $Y_{Ethanol\text{-}Process}$: overall process ethanol yield; YPD: yeast extract peptone glucose; ηHandling: efficiency factor of conditioning steps; ηGlucose/Xylose: conversion efficiency of enzymatic hydrolysis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VN and BN designed the research. VN planned the experiments. VN and KL performed the experiments and analyzed the data. The manuscript was written from contributions of all authors. VN and BN wrote the paper. The manuscript has been read and approved by all authors.

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References

1. Gnansounou E. Production and use of lignocellulosic bioethanol in Europe: current situation and perspectives. Bioresour Technol. 2010;101:4842–50.
2. Gnansounou E, Dauriat A. Techno-economic analysis of lignocellulosic ethanol: a review. Bioresour Technol. 2010;101:4980–91.
3. Chen H, Qiu W. Key technologies for bioethanol production from lignocellulose. Biotechnol Adv. 2010;28:556–62.
4. Sukumaran RK, Surender VJ, Sindhu R, Binod P, Janu KU, Sajna KV, et al. Lignocellulosic ethanol in India: prospects, challenges and feedstock availability. Bioresour Technol. 2010;101:4826–33.
5. Cardona CA, Sánchez ÓJ. Fuel ethanol production: process design trends and integration opportunities. Bioresour Technol. 2007;98:2415–57.
6. Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G. Bio-ethanol – the fuel of tomorrow from the residues of today. Trends Biotechnol. 2006;24:549–56.
7. Govumoni SP, Koti S, Kothagouni SY, Venkateshwar S, Linga VR. Evaluation of pretreatment methods for enzymatic saccharification of wheat straw for bioethanol production. Carbohydr Polym. 2013;91:646–50.
8. Sims REH, Mabee W, Saddler JN, Taylor M. An overview of second generation biofuel technologies. Bioresour Technol. 2010;101:1570–80.
9. Talebnia F, Karakashev D, Angelidaki I. Production of bioethanol from wheat straw: an overview on pretreatment, hydrolysis and fermentation. Bioresour Technol. 2010;101:4744–53.
10. van Maris A, Abbott D, Bellissimi E, van den Brink J, Kuyper M, Luttik M, et al. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Van Leeuwenhoek. 2006;90:391–418.
11. Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol. 2010;101:4851–61.
12. Erdei B, Barta Z, Sipos B, Reczey K, Galbe M, Zacchi G. Ethanol production from mixtures of wheat straw and wheat meal. Biotechnol Biofuels. 2010;3:16.
13. Olofsson K, Palmqvist B, Liden G. Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding. Biotechnol Biofuels. 2010;3:17.
14. Sassner P, Galbe M, Zacchi G. Techno-economic evaluation of bioethanol production from three different lignocellulosic materials. Biomass Bioenergy. 2008;32:422–30.
15. Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund M. Towards industrial pentose-fermenting yeast strains. Appl Microbiol Biotechnol. 2007;74:937–53.
16. Matsushika A, Inoue H, Kodaki T, Sawayama S. Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. Appl Microbiol Biotechnol. 2009;84:37–53.
17. Viikari L, Vehmaanperä J, Koivula A. Lignocellulosic ethanol: from science to industry. Biomass Bioenergy. 2012;46:13–24.
18. Klimacek M, Kirl E, Krahulec S, Longus K, Novy V, Nidetzky B. Stepwise metabolic adaption from pure metabolization to balanced anaerobic growth on xylose explored for recombinant *Saccharomyces cerevisiae*. Microb Cell Fact. 2014;13:37.
19. Novy V, Krahulec S, Wegleiter M, Muller G, Longus K, Klimacek M, et al. Process intensification through microbial strain evolution: mixed glucose-xylose fermentation in wheat straw hydrolyzates by three generations of recombinant *Saccharomyces cerevisiae*. Biotechnol Biofuels. 2014;7:49.
20. Petschacher B, Nidetzky B. Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. Microb Cell Fact. 2008;7:9.
21. Chundawat SPS, Beckham GT, Himmel ME, Dale BE. Deconstruction of lignocellulosic biomass to fuels and chemicals. Annu Rev Chem Biomol Eng. 2011;2:121–45.
22. Macrelli S, Mogensen J, Zacchi G. Techno-economic evaluation of 2nd generation bioethanol production from sugar cane bagasse and leaves integrated with the sugar-based ethanol process. Biotechnol Biofuels. 2012;5:22.
23. Larsen J, Østergaard Petersen M, Thirup L, Wen Li H, Krogh IF. The IBUS process – lignocellulosic bioethanol close to a commercial reality. Chem Eng Technol. 2008;31:765–72.
24. Maas RH, Bakker RR, Boersma AR, Bisschops I, Pels JR, de Jong E, et al. Pilot-scale conversion of lime-treated wheat straw into bioethanol: quality assessment of bioethanol and valorization of side streams by anaerobic digestion and combustion. Biotechnol Biofuels. 2008;1:14.
25. Erdei B, Franko B, Galbe M, Zacchi G. Separate hydrolysis and co-fermentation for improved xylose utilization in integrated ethanol production from wheat meal and wheat straw. Biotechnol Biofuels. 2012;5:12.
26. Sorensen A, Teller PJ, Lubeck PS, Ahring BK. Onsite enzyme production during bioethanol production from biomass: screening for suitable fungal strains. Appl Biochem Biotechnol. 2011;164:1058–70.
27. Novy V, Krahulec S, Longus K, Klimacek M, Nidetzky B. Co-fermentation of hexose and pentose sugars in a spent sulfite liquor matrix with genetically modified *Saccharomyces cerevisiae*. Bioresour Technol. 2013;130:439–48.
28. Kim S, Dale B. Global potential bioethanol production from wasted crops and crop residues. Biomass Bioenergy. 2004;4:361–75.
29. Peterson R, Nevalainen H. *Trichoderma reesei* RUT-C30–thirty years of strain improvement. Microbiology. 2012;158:58–68.
30. Schuster A, Schmoll M. Biology and biotechnology of *Trichoderma*. Appl Microbiol Biotechnol. 2010;87:787–99.
31. Esterbauer H, Steiner W, Labudova I, Hermann A, Hayn M. Production of *Trichoderma* cellulase in laboratory and pilot scale. Bioresour Technol. 1991;36:51–65.
32. Persson I, Tjerneld F, Hahn-Hägerdal B. Fungal cellulolytic enzyme production: a review. Process Biochem. 1991;26:65–74.
33. Mandels M, Reese ET. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J Bacteriol. 1957;73:269–78.
34. Chen M, Qin Y, Liu Z, Liu K, Wang F, Qu Y. Isolation and characterization of a β-glucosidase from *Penicillium decumbens* and improving hydrolysis of corncob residue by using it as cellulase supplementation. Enzyme Microb Technol. 2010;46:444–9.
35. Ma L, Zhang J, Zou G, Wang C, Zhou Z. Improvement of cellulase activity in *Trichoderma reesei* by heterologous expression of a beta-glucosidase gene from *Penicillium decumbens*. Enzyme Microb Technol. 2011;49:366–71.
36. Murray P, Aro N, Collins C, Grassick A, Penttila M, Saloheimo M, et al. Expression in *Trichoderma reesei* and characterisation of a thermostable family 3 beta-glucosidase from the moderately thermophilic fungus *Talaromyces emersonii*. Protein Expr Purif. 2004;38:248–57.
37. Nakazawa H, Kawai T, Ida N, Shida Y, Kobayashi Y, Okada H, et al. Construction of a recombinant *Trichoderma reesei* strain expressing *Aspergillus aculeatus* beta-glucosidase 1 for efficient biomass conversion. Biotechnol Bioeng. 2012;109:92–9.
38. Gruno M, Väljamäe P, Pettersson G, Johansson G. Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate. Biotechnol Bioeng. 2004;86:503–11.
39. Murphy L, Bohlin C, Baumann MJ, Olsen SN, Sørensen TH, Anderson L, et al. Product inhibition of five *Hypocrea jecorina* cellulases. Enzyme Microb Technol. 2013;52:163–9.
40. Ahamed A, Vermette P. Effect of culture medium composition on *Trichoderma reesei*'s morphology and cellulase production. Bioresour Technol. 2009;100:5979–87.
41. Domingues FC, Queiroz JA, Carbral JMS, Fonseca LP. The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. Enzyme Microb Technol. 2000;26:394–401.
42. Clariant AG. Sunliquid® - Cellulosic ethanol from agricultural residues. [http://www.clariant.com/en/Solutions/Products/2014/10/16/16/16/sunliquid] Accessed 22 Jan 2015.
43. Weiss N, Borjesson J, Pedersen LS, Meyer AS. Enzymatic lignocellulose hydrolysis: improved cellulase productivity by insoluble solids recycling. Biotechnol Biofuels. 2013;6:5.
44. Yang J, Zhang X, Yong Q, Yu S. Three-stage hydrolysis to enhance enzymatic saccharification of steam-exploded corn stover. Bioresour Technol. 2010;101:4930–5.

45. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, et al. Determination of structural carbohydrates and lignin in biomass. National Renewable Energy Laboratory: Golden, CO, USA; 2011.
46. Ghose T. Measurement of cellulase activities. Pure Appl Chem. 1987;2:257–68.
47. Takashima S, Nakamura A, Hidaka M, Masaki H, Uozumi T. Molecular cloning and expression of the novel fungal beta-glucosidase genes from *Humicola grisea* and *Trichoderma reesei*. J Biochem. 1999;125:728–36.
48. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.

Chapter 4

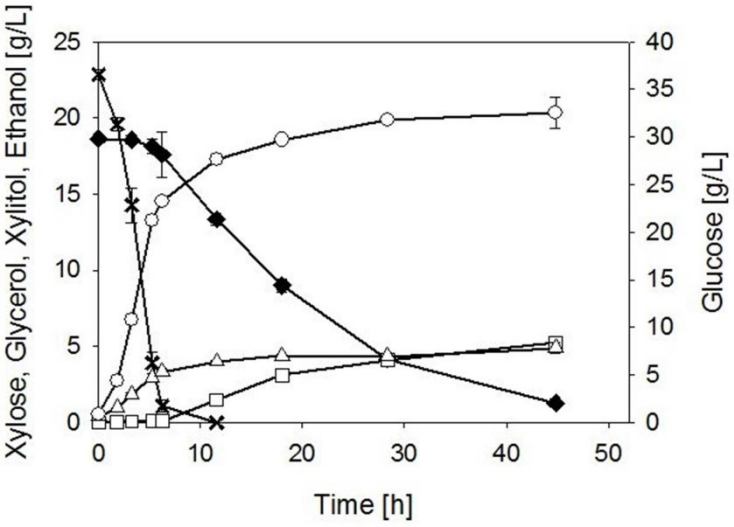
Supplementary information

Additional file 1: SI-1

Mixed glucose-xylose fermentation in 15% hydrolyzate utilizing *S. cerevisiae* strain IBB10B05

Additional file 2: SI-2

Summary of in- and output parameters and mass balance analyses of the different process configurations



Additional file 1: Mixed glucose-xylose fermentation in 15% hydrolyzate utilizing *S. cerevisiae* strain IBB10B05. Time course was derived from a previous publication [19]. Symbols: glucose (crosses), xylose (full diamonds), glycerol (empty triangles), xylitol (empty squares), ethanol (empty circles).

Additional file 2: Summary of in- and output parameters and mass balance analyses of the different process configurations.

	1st step	2nd step		3rd step		Input - pre-treated substrate				Input - raw material	Output	
	[FPU/mL]	[FPU/$g_{DM\ WS}$]	η Glucose [%]	η Xylose [%]	Y Ethanol	η Handling WS [%]	pre-culture WS	fungal ferm WS	enz hydrolysis WS	Total [g DM WS]	Total [g DM RM]	Ethanol [g]	$Y_{Ethanol\text{-}Process}$ [$g_{Ethanol}$/$kg_{DM\ RM}$]
Config.1 - "benchtop process"	1.7	30.0	67.0	95.0	0.4 g/gGlc+Xyl	75.0	5.6	120.0	170.0	295.6	325.2	23.1	71.2
Config.2 - "fed-batch"	2.7	30.0	67.0	95.0	0.4 g/gGlc+Xyl	75.0	5.6	300.0	270.0	575.6	633.2	36.7	58.0
Config.3 - "25 FPU/g DM WS"	1.7	25.0	60.0	81.0	0.4 g/gGlc+Xyl	75.0	5.6	120.0	204.0	329.6	362.6	24.5	67.5
Config.4 - "no xylose conversion"	1.7	30.0	67.0	95.0	0.45 g/gGlc	75.0	5.6	120.0	170.0	295.6	325.2	17.8	54.6
Config.5 - "no enzyme production"	-	30.0	67.0	95.0	0.4 g/gGlc+Xyl	75.0	-	-	170.0	170.0	187.0	23.1	123.7
Config.6 - "recycling"	1.7	30.0	90.0	95.0	0.4 g/gGlc+Xyl	75.0	5.6	120.0	170.0	295.6	325.2	28.6	87.8
Config.7 - "beta-glucosidase"	1.7	30.0	78.0	100.0	0.4 g/gGlc+Xyl	75.0	5.6	120.0	170.0	295.6	325.2	26.1	80.3
Config.8 - "theoretical max."	1.7	30.0	100.0	100.0	0.51 g/gGlc+Xyl	100.0	5.6	120.0	226.7	352.3	387.5	70.9	183.1

Scientific record

Publications

Novy, V., Longus, K., & Nidetzky, B. (2015). From wheat straw to bioethanol: integrative analysis of a separate hydrolysis and co-fermentation process with implemented enzyme production. *Biotechnol. Biofuels*, *8*(1), 46.

Novy, V., Krahulec, S., Wegleiter, M., Müller, G., Longus, K., Klimacek, M., & Nidetzky, B. (2014). Process intensification through microbial strain evolution: mixed glucose-xylose fermentation in wheat straw hydrolyzates by three generations of recombinant *Saccharomyces cerevisiae*. *Biotechnol. Biofuels*, *7*, 49.

Klimacek, M., Kirl, E., Krahulec, S., Longus, K., **Novy, V.**, & Nidetzky, B. (2014). Stepwise metabolic adaption from pure metabolization to balanced anaerobic growth on xylose explored for recombinant *Saccharomyces cerevisiae*. *Microb. Cell Fact.*, *13*, 37.

Novy, V., Krahulec, S., Longus, K., Klimacek, M., & Nidetzky, B. (2013). Co-fermentation of hexose and pentose sugars in a spent sulfite liquor matrix with genetically modified *Saccharomyces cerevisiae*. *Bioresour. Technol.*, *130*, 439-448.

Oral presentations

Co-fermentation of hexose and pentose sugars for bioethanol production in lignocellulosic feedstock using a genetically modified *Saccharomyces cerevisiae*

V. Novy, S. Krahulec, K. Longus and B. Nidetzky (2013)

9th European Congress of Chemical Engineering, The Hague, Netherlands

From lignocellulosic waste streams to bioethanol - An integrated approach utilizing a genetically optimized *Saccharomyces cerevisiae* strain

V. Novy and B. Nidetzky (2014)

10th European Symposium on Biochemical Engineering Sciences and 6th International Forum on Industrial Bioprocesses, Lille, France

From wheat straw to bioethanol: Integrative analysis of a SHCF process with implemented enzyme production

V. Novy and B. Nidetzky (2015)

37th Symposium on Biotechnology for Fuels and Chemicals, San Diego, USA