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Posted Date: 21 September 2023

doi: 10.20944/preprints202309.1412.v1

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Oxidative Phosphorylation for Aerobic Survival, But Not for Growth: The Peculiar 'Make-Accumulate-Consume' Strategy in *Zymomonas mobilis*

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Abstract: Understanding the energy metabolism and its regulation is one of the clues to metabolic engineering of stress-resistant lignocellulose-converting microbial strains, including also the promising ethanologen *Zymomonas mobilis*. *Z. mobilis* is an obligately fermentative, facultatively anaerobic bacterium, carrying an active respiratory chain with a low energy-coupling efficiency. Its respiration does not supply energy to aerobically growing culture on sugary media, yet oxidative phosphorylation has been demonstrated in non-growing cells with ethanol. In the present work we show that in respiring, non-growing *Z. mobilis* cells, receiving regular small amounts of ethanol, oxidative phosphorylation significantly contributes to the maintenance of their viability. Accordingly, no improvement of viability is seen in the NADH dehydrogenase (*ndh*)-deficient respiratory mutant, unable to oxidize ethanol. The ethanol effect is hampered also by the protonophoric uncoupler CCCP, or the inhibitor of ATP synthase, DCCD. At higher concentrations (6% v/v) ethanol causes stress that slows down culture growth. By monitoring the activity of several respiratory gene promoters in aerobically growing culture under ethanol stress with the green fluorescent protein reporter system, we demonstrate downregulation of these promoters, in particular the *ndh* promoter. We speculate that the decrease of the respiratory chain activity mitigates the production of reactive oxygen species.

Keywords: *Zymomonas mobilis*; respiratory chain; Type II NADH dehydrogenase; oxidative phosphorylation; starvation; viability; make-accumulate-consume strategy; ethanol stress

1. Introduction

Zymomonas mobilis is an ethanologenic, facultatively anaerobic alpha-proteobacterium. It posesses the most rapid homoethanol fermentation pathway among microorganisms [1], and apart from that, it can tolerate high sugar and ethanol concentrations [2,3]. These properties, in combination with a small genome size and relatively simple central metabolism have put *Z. mobilis* in the focus of metabolic engineering work on production of bioethanol and several other commodity chemicals from renewable substrates, primarily from lignocellulose hydrolysates [4–8]. Lignocellulose hydrolysates contain several types of inhibitory compounds, therefore resistance to inhibitor stress, and more generally, robustness of producer strains is critical for lignocellulose bioconversion [5]. Generally speaking, robustness results from a complex network of physiological properties, yet in bacteria much of it is related to cellular energy management, and more specifically, to the function of respiratory chain [9–11] that has been shown to impact various types of stress resistance [12–14].

 doi:10.20944/preprints202309.1412.v1

Understanding the respiratory energy metabolism and its regulation is one of the clues to metabolic engineering of stress-resistant lignocellulose-converting microbial strains.

While the fermentative energy generation in *Z. mobilis* takes place in the Entner-Doudoroff pathway, and is fairly well understood, it is not so with its respiratory energetics. *Z. mobilis* bears a constitutive respiratory chain (see Figure 1). Its major electron pathway involves the Type II NADH dehydrogenase, coenzyme Q10, and the cytochrome *bd* terminal oxidase. FAD-dependent D-lactate dehydrogenase and PQQ-dependent glucose dehydrogenase (Gdh) are minor dehydrogenase activities [15–18]. The cytochrome *bd* is the only terminal oxidase, encoded in the genome [19,20], and detected spetroscopically [16,18]. The respiratory peroxidase, identified in the genome as a cytochrome *c* peroxidase (PerC), most likely functions as a quinol peroxidase [21]. The cytochrome bc_1 branch of electron transport seems to be a 'dead-end', lacking a terminal cytochrome *c* oxidase, and hence, its function remains obscure.

For some unknown reason, the energetic efficiency of *Z. mobilis* respiration in aerobically growing culture is much lower than in other bacteria (e.g., *L. lactis*) that employ a similar electron transport route from the Type II NADH dehydrogenase to cytochrome *bd* terminal oxidase [9,22,23]. Although oxidative phosphorylation capacity of *Z. mobilis* respiratory chain has been demonstrated with non-growing cells and membrane vesicles [24], aeration does not contribute to its biomass yield [25], (for a review, see [26]), making its aerobic energetics quite unique among the known biotechnological producer microorganisms. Furthermore, respiration hampers its aerobic growth by disturbing the fermentative redox balance. In the absence of a functional TCA cycle, respiratory chain withdraws NADH mostly from the alcohol dehydrogenase reaction, thus causing accumulation of the inhibitory metabolite acetaldehyde. Turning down the *Z. mobilis* respiration decreases acetaldehyde accumulation, and improves aerobic growth and ethanol synthesis, as seen in the *ndh* knock-out mutant growing on rich medium [17,27–29]. Complementation of this mutant strain with an Ndh-expressing plasmid vector restores its respiratory activity, and accordingly, its aerobic growth decreases to the level of the parent strain. These observations highlight a problem: for what purpose *Zymomonas mobilis* has preserved its respiratory chain, given that it is inhibitory for its own aerobic growth?

To understand the physiological rationale behind *Z. mobilis* respiration, it is helpful to compare its metabolism with other ethanologenic microorganisms, primarily with that of yeast. Part of yeast species, including *Saccharomyces cerevisiae*, are Crabtree-positive, which means that in sugar-rich environments they perform fermentation along with respiration even under vigorous oxygenation [30]. Consuming sugar faster than other species, converting it to ethanol to inhibit the growth of competing bacteria, and then taking up the accumulated ethanol as the carbon and energy substrate after they have established dominance in their niche, is a growth strategy termed 'make-accumulate-consume' (MAC) [31,32]. *Z. mobilis* converts sugar to ethanol even faster than the ethanologenic yeast [1], and is equally resistant to high ethanol concentration. However, it is an obligately fermentative bacterium that can oxidize ethanol, but unlike yeast, cannot use it as a carbon source for growth [3]. Since respiration with ethanol in non-growing cells produces ATP via oxidative phosphorylation [24], we hypothesize that the *Z. mobilis* version of MAC strategy might be to employ their accumulated ethanol merely as an oxidizable energy substrate, used to maintain viability of non-growing cells. The MAC strategy can have also its disadvantages: although ethanol inhibits growth of competing microorganisms and serves as an oxidizable energy source, at higher concentration it causes stress to the producer organism itself [33,34]. How the respiratory chain functions under condition when *Z. mobilis* (if grown on a sugar rich media) accumulates high amounts of ethanol, is thus another question related to its putative MAC strategy. In the present study we demonstrate that ethanol can serve as a respiratory energy source for maintenance of viability, yet under ethanol stress condition *Z. mobilis* partly turns down its respiratory chain activity.

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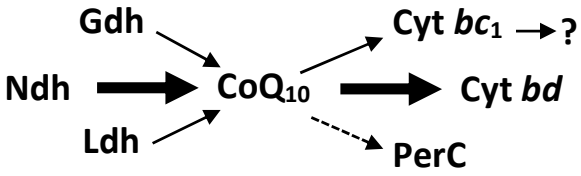

Figure 1. Electron transport chain of *Zymomonas mobilis*. Ndh, type II NADH dehydrogenase; Ldh, lactate dehydrogenase; Gdh, glucose dehydrogenase; PerC, cytochrome *c* peroxidase; cyt *bd*, cytochrome *bd* terminal oxidase; Cyt bc_1, cytochrome bc_1 complex.

2. Materials and Methods

2.1. Plasmid and Strain Construction

The plasmids and strains used in this study are listed in Table 1. For monitoring of promoter activity of the *Z. mobilis* respiratory genes, reporter systems with engineered green fluorescent protein (EGFP) were constructed. The gene *egfp* was amplified by PCR, taking plasmid pQE-EGFP as the template. The PCR product was double-digested with HindIII and EcoRI restriction enzymes, and inserted in the MCS of pBBR1MCS-2 between the respective sites, yielding plasmid pBBR-EGFP. After that, chromosomal DNA sequences of the strain Zm6 (ATCC 29191), containing the promoter regions of cytochrome *c* peroxidase, cytochrome bc_1 operon, cytochrome *bd* operon, and the type II NADH dehydrogenase, were amplified. Sequence data of the amplified DNA fragments and the corresponding primers are given in the Table A1. These PCR products were double-digested and inserted in pBBR-EGFP directly upstream of *egfp*, between the EcoRI and BamHI sites of its MCS, yielding the four reporter plasmids listed in Table 1. Genomic DNA isolation from *Z. mobilis*, PCR assays, as well as DNA purification and cloning techniques were the same as described before [17,35]. *Escherichia coli* strain DH5α was used for plasmid cloning. The obtained reporter plasmid constructs were verified by restriction analysis, and by PCR (not shown), using primer pairs consisting of an F primer for the respective promoter region and R primer for *egfp* (see Table A1). Finally, *Z. mobilis* Zm6 was transformed with pBBR-EGFP and with the four reporter plasmids by electroporation [36]. Successful transformations were verified by colony PCR.

Table 1. Plasmids and strains used in this study.

Plasmid/Strain	Characteristics	Source
pBBR1MCS-2	Plasmid vector, kan^r	Addgene
pNdh	pBBR1MCS-2 derivative with a 1.55 kb insert between the HindIII and BamHI sites of its MCS, containing the ORF of the type II respiratory NADH dehydrogenase (*ndh*, gene ZZ6_0213) together with its native promoter	[23]
pQE-EGFP	pQE-30 plasmid with an 0.72 kb insert of EGFP coding sequence in the MCS	Addgene
pBBR-EGFP	pBBR1MCS-2 derivative with an 0.72 kb insert of EGFP coding sequence in the MCS between EcoRI and HindIII restriction sites	Present work
pBBR:P*ndh*-EGFP	pBBR-EGFP derivative with an 0.26 kb insert, containing the promoter region of the type II respiratory NADH dehydrogenase (*ndh*, gene ZZ6_0213), upstream of the EGFP coding sequence in the MCS between BamHI and EcoRI restriction sites	Present work

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pBBR:P*bc*$_1$-EGFP	pBBR-EGFP derivative with an 0.23 kb insert, containing the promoter region of the cytochrome *bc*$_1$ operon (genes ZZ6_0349, ZZ6_0348, ZZ6_0347), upstream of the EGFP coding sequence in the MCS between BamHI and EcoRI restriction sites	Present work
pBBR:P*bd*-EGFP	pBBR-EGFP derivative with an 0.76 kb insert, containing the promoter region of the cytochrome *bd* operon (*cydAB*; genes ZZ6_1532, ZZ6_1531), upstream of the EGFP coding sequence in the MCS between BamHI and EcoRI restriction sites	Present work
pBBR:P*perC*-EGFP	pBBR-EGFP derivative with an 0.42 kb insert, containing the promoter region of the cytochrome *c* peroxidase (*perC*, gene ZZ6_0192), upstream of the EGFP coding sequence in the MCS between BamHI and EcoRI restriction sites	Present work
Zm6	Wild type, parent strain	ATCC 29191
Zm6-*ndh*	Zm6 with a Cm^r (chloramphenicol acetyltransferase gene) insert in the AgeI site of the chromosomal ORF of its *ndh*	[17]
Zm6-*ndh*_pNdh	Zm6-*ndh* transformed with plasmid pNdh	[23]
Zm6_ pBBR-EGFP	Zm6 transformed with plasmid pBBR-EGFP	Present work
Zm6_ pBBR:P*ndh*-EGFP	Zm6 transformed with plasmid pBBR:P*ndh*-EGFP	Present work
Zm6_ pBBR:P*bc*$_1$-EGFP	Zm6 transformed with plasmid pBBR:P*bc*$_1$-EGFP	Present work
Zm6_ pBBR:P*bd*-EGFP	Zm6 transformed with plasmid pBBR:P*bd*-EGFP	Present work
Zm6_ pBBR:P*perC*-EGFP	Zm6 transformed with plasmid pBBR:P*perC*-EGFP	Present work

2.2. Cultivation, Starvation and Assessment of Viability

Z. mobilis strains were maintained and cultivated at 30 °C, without aeration, on medium containing (per 1 L of distilled water) 50 g glucose, 5 g yeast extract, 1 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, and 0.5 g of $MgSO_4 \times 7H_2O$ [24]. Overnight cultivations of mutant / recombinant strains carrying antibiotic resistance markers were done with added antibiotics (chloramphenicol, 120 μg mL^{-1} and/or kanamycin, 310 μg mL^{-1}, where required) [23,37]. For *Z. mobilis* electroporation, RM growth medium containing (per 1 L of distilled water) 20 g glucose, 10 g yeast extract and 2 g KH_2PO_4 was used. *E. coli* DH5α was grown on LB medium, containing (per liter) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl. Medium for plasmid-bearing *E. coli* strains was supplemented with 60 μg mL^{-1} of kanamycin. For platings, all media were supplemented with 2 g L^{-1} of agar.

For starvation experiments, the following three strains (Table 1): (i) the wild type Zm6, (ii) the Ndh knock-out mutant strain Zm6-*ndh*, and (iii) the mutant strain complemented with a plasmid vector, carrying *ndh* under its native promoter, Zm6-*ndh*_pNdh, were cultivated overnight without shaking in a thermostat at 30 °C. The cells were harvested by centrifugation, washed and resuspended in 0.1 M potassium phosphate buffer (pH 7), supplemented with 5 mM $MgSO_4$, to yield OD_{550} around 10 ± 0.5 (equivalent to 2 mg dry wt mL^{-1}). 10 mL of cell suspensions were transferred into 50 mL shaken flasks, and incubated on a shaker at 100 r.p.m., 30 °C, for 6 hours. To assess the cell viability, 50 μL samples were taken from the flasks at time zero and after the 6 h period. These samples were diluted with distilled water to OD_{550} of 0.5, and for each experimental condition, four technical replicates of serial dilutions (from 10^0 to 10^{-4}) were produced and spotted on agar plates in 5 μL aliquots. To determine statistical significance of the difference between the outcomes of various

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experiment series, experiments where compared using the nonparametric Mann Whitney U test (Wilcoxon Rank Sum test) based on ranking the growth on the plates.

2.3. Cultivation of Egfp-Bearing Strains in the Microplate Reader and Monitoring EGFP Fluorescence

The EGFP-bearing *Z. mobilis* strains were cultivated in a microplate (96-well plate) reader Infinite M200 PRO Multimode Microplate Reader (Tecan, Maennedorf, Switzerland) at 30 °C, for 15 hours. Each well contained 200 µl of culture. The plates were shaken at 200 r.p.m., with 3.5 mm amplitude, in 490 s periods with interruptions for measurements. Optical density (at 600 nm) and fluorescence (excitation at 475 nm, emission at 510 nm) measurements were taken at 10.5 min intervals. Each experiment was carried out in at least 2 technical replicates.

2.4. FTIR Spectroscopy of Biomass

FTIR spectra of *Z. mobilis* biomass were recorded using Vertex 70 coupled with the microplate reader HTS-XT (Bruker, Germany), as described in [38]. In brief, spectra were recorded in the frequency range of 4000–600 cm^{-1}, with a spectral resolution of 4 cm^{-1}, and 64 scans were coadded. Only spectra with absorbance within the absorption limits between 0.30 and 1.00 (where the concentration of a component is proportional to the intensity of the absorption band) were used for analysis. Data were processed using OPUS 7.5 software (Bruker Optics GmbH, Ettlingen, Germany). The baseline of each spectrum was corrected by the rubber band method. The macromolecular composition of biomass was calculated as described previously [39].

2.5. Preparation of Cytoplasmic Membranes and the Respiratory Chain NADH Oxidase Assay

For assaying the respiratory NADH oxidase activity, cytoplasmic membrane vesicles were prepared from cells, grown aerobically in 250 mL shaken flasks (with 100 mL culture volume) at 100 r.p.m., 30 °C, and harvested after 8 hours of cultivation, during their transition from the late exponential to early stationary growth phase. After sedimentation, washing and resuspension in 0.1 M potassium phosphate buffer with 5 mM $MgSO_4$, pH 7, followed by ultrasonic breakage and removal of unbroken cells, the cell membrane fraction was sedimented by ultracentrifugation, basically following the procedures described previously [37]. Sedimentation was conducted in a Thermo Scientific Sorvall WX+ Ultracentrifuge at 35,000 r.p.m. for 1 h, the pellet was washed with phosphate buffer, membranes were resuspended, and centrifuged repeatedly for 1 h at the same speed. Finally, the pellet was resuspended in the same buffer to yield membrane vesicle suspension with protein concentration in the range of 3 – 4 mg mL^{-1}. Protein concentration was determined as previously described [37]. 1 mL of NADH oxidase assay mixture contained 980 µl 0.1 M phosphate buffer, pH 7, 10 µl of 10mM NADH and 10 µl of membrane vesicle suspension. NADH oxidase activity was measured by monitoring NADH absorbance decrease at 340 nm, taking 6.22 $mM^{-1}cm^{-1}$ as the millimolar extinction coefficient.

3. Results

3.1. The Impact of Respiratory Chain Activity on the Viability of Non-Growing Z. mobilis Cells

Already during the early stage of research in *Z. mobilis* (formerly, also *Z. anaerobia*; see [40]) physiology, Dawes and Large [41] showed that in starving cell population "viability decreased exponentially as soon as starvation began, as might be expected since *Zymomonas anaerobia* contains no energy reserves to protect against starvation. The decrease in viability was accompanied by an exponential decrease in intracellular ATP which was most marked during the initial 6 hr." Our FTIR spectroscopy data on the macromolecular constituents of the parent strain Zm6 before and after a 6 hour starvation period (Table B1) supported the notion that *Z. mobilis* cells did not employ endogenous energy reserves: the spectra revealed fairly constant relative amounts of the bulk intracellular carbohydrate, nucleic acids, lipids and protein. Accordingly, we concluded that viability should mostly depend on external energy source. For *Z. mobilis*, when run out of carbohydrates in its

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growth medium, ethanol might be the energy source of choice. We here chose the 6 hour starvation period to examine, if ethanol oxidation could be used for energy production to maintain viability of non-growing, respiring *Z. mobilis* cells.

The results are presented in the Figure 2 A. Without supply of external energy source, there was a significant drop of viability after the 6 hour starvation for all strains. In the experiment series '6h+glucose' the cell suspensions once per hour were supplemented with small amounts of glucose, yielding 1 g L^{-1} its concentration in the medium. Glucose was taken up within approx. 15 minutes (not shown), and hence, most of the time cells were starving. Nevertheless, for all strains this amount of energy substrate proved sufficient to maintain their viability. In the experiment series '6h+ethanol' cells with the same regularity were supplied with ethanol in 5 g L^{-1} increments. Regular ethanol addition maintained viability in the respiring strains Zm6 and Zm6-*ndh*_pNdh to the same extent as did glucose supplements, but in Zm6-*ndh* ethanol had no positive effect – in a striking contrast to the aerobic growth on rich medium (Figure 2 B), where the mutant Zm6-*ndh* by far outperformed the other two strains.

We have shown previously that non-growing respiring cells of *Z. mobilis* can perform oxidative phosphorylation using ethanol as substrate [24]. The present finding strongly indicates that in *Z. mobilis* strains, which are able to oxidize ethanol, oxidative phosphorylation can serve as the energy source for maintenance of their viability. Because of the lack of NADH dehydrogenase in its respiratory chain, the mutant Zm6-*ndh* cannot oxidize ethanol, and accordingly it cannot use ethanol for energy production via oxidative phosphorylation to maintain its viability. With glucose, on the other hand, the bulk of ATP comes from the fermentative catabolism that is roughly similar in all three strains.

To reinforce the proposed role of oxidative phosphorylation for the maintenance of viabilty, we performed inhibitor analysis of the ethanol effect with the F_1F_o-ATP synthase inhibitor N,N'-dicyclohexylcarbodiimide, DCCD (Figure 2 A, series '6h+ethanol+DCCD) and the protonophoric uncoupler carbonyl cyanide m-chlorophenylhydrazone, CCCP (series '6h+ethanol+CCCP). DCCD at 50 µM final concentration, or CCCP at 10 µM final concentration, were added to the cell suspensions shortly before the start of incubations with the periodical ethanol supply. For the strains Zm6 and Zm6-*ndh*_pNdh, these inhibitors hampered the ethanol effect. A decrease of viability was seen with both inhibitors and in both strains. Yet, the most pronounced, statistically significant effect ($P<0.05$) was obtained for Zm6 with CCCP.

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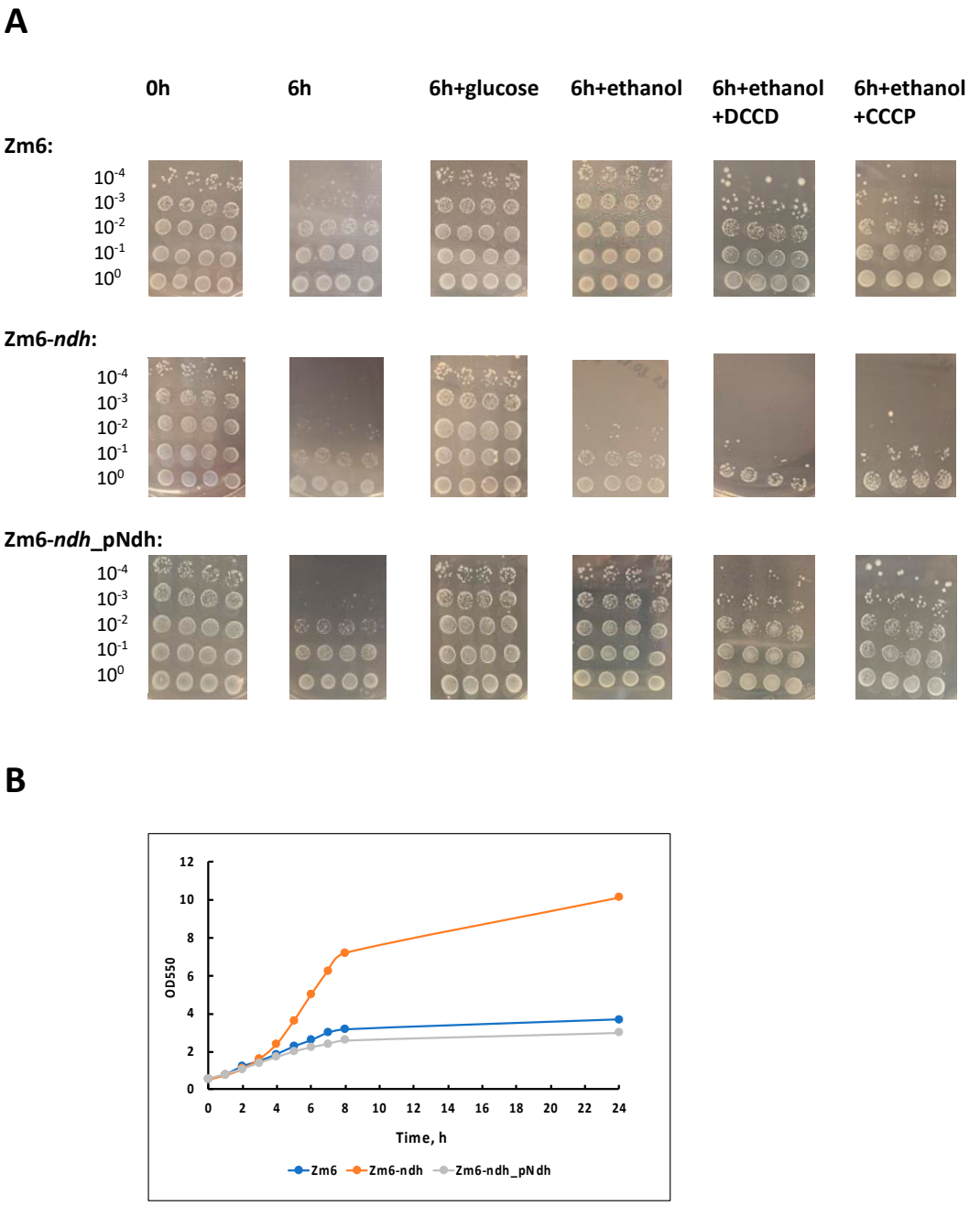

Figure 2. Viability of aerobically incubated non-growing cells (A) and aerobic batch growth (B) of the strains Zm6, Zm6-*ndh* and Zm6-*ndh*_pNdh. In (A), a representative set of spot tests is shown. The 6 hour starvation experiment, and incubations with addition of glucose or ethanol were done in four biological replicates (each with 4 technical repeats); incubations with added DCCD or CCCP had three biological replicates. According to the Mann Whitney U test, statistically significant difference ($P<0.05$) in viability were observed between following samples: 0h vs. 6h for all strains; 6h vs. 6h+glucose for all strains; 6h vs. 6h+ethanol for Zm6 and Zm6-*ndh*_pNdh; 0h (or 6h+glucose) vs. 6h+ethanol (also, 6h+ethanol+DCCD, or +CCCP) for Zm6-*ndh*; 6h+ethanol for Zm6-*ndh* vs. 6h+ethanol for the other two strains; 6h+ethanol vs. 6h+ethanol+CCCP for Zm6. (B), a representative time-course of aerobic batch growth of the strains in 200 mL shaken flasks with 25mL of culture, at 180 r.p.m. and 30 °C.

3.2. Expression of the Respiratory Chain Complexes under Ethanol Stress

As we show here, at low ethanol concentrations respiring *Z. mobilis* uses it as an energy source for maintenance of viabilty. Yet, at higher concentrations, ethanol causes stress by inhibiting culture

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growth and metabolism. We assumed that monitoring the ethanol stress-induced changes of the expression pattern of *Z. mobilis* respiratory chain modules might help to draw conclusions about their involvement in ethanol stress protection. In order to see how the respiratory chain components responded to ethanol stress, we examined recombinant strains carrying the EGFP reporter plasmids with four respiratory gene promoters (see Table 1). Ethanol stress was induced by addition of ethanol to growth medium at 6% (v/v) concentration, as described elsewhere [42,43], and cultivation was carried out in a microplate reader allowing simultaneous recording of the culture OD and fluorescence. Ethanol at this concentration already significantly hampered culture growth (Figure 3, inset).

EGFP fluorescence data in the Figure 3 correspond to the 8th hour of cultivation in the microplate reader, when cultures were entering early stationary growth phase. First, for each strain the recorded fluorescence value was normalized per unit of its culture optical density. Since the normalized fluorescence of the strain Zm6_ pBBR-EGFP was identical to that of Zm6 throughout the whole range of culture OD values (not shown), we concluded that EGFP did not get expressed from the plasmid pBBR-EGFP. The normalized fluorescence of Zm6_ pBBR-EGFP was therefore taken for baseline, implying all fluorescence exceeding that level in the four other EGFP-bearing strains to result from the activity of the respective respiratory gene promoters. Accordingly, the 'normalized fluorescence ratio' shown in the Figure 3 denotes the ratio of the normalized fluorescence of any promoter-carrying strain to the normalized fluorescence of strain Zm6_ pBBR-EGFP. A ratio of 1.0 in this scale would mean a complete lack of promoter activity.

As seen from the Figure 3, under non-stressed conditions all promoters were active. For all of them the normalized fluorescence ratio was significantly above 1.0 ($P<0.05$). The *ndh* promoter, however, stood out by being several times more active than any of the three other promoters. Under ethanol stress its activity was reduced by more than half, while only a slight (statistically not significant) decrease was seen for the rest of promoters. The *perC* promoter was the weakest of all. Its normalized fluorescence ratio was the closest to 1.0 already under non-stressed condition, but under ethanol stress it showed no statistically significant activity. For the *ndh* promoter our data fell in line with previously published transcriptomic findings, demonstrating downregulation of *ndh* under ethanol stress [42]. Yet, we did not see any significant effects with the cytochrome *bd* and cytochrome bc_1 promoters, although upregulation of *bd* and downregulation of bc_1 transcripts was reported in the same study.

NADH oxidase activity in the membrane preparation of non-stressed Zm6_ pBBR-EGFP was 0.64 (±0.08) U mg prot^{-1}, while under ethanol stress it was down to only 0.28 (±0.05) U mg prot^{-1}. This corresponded well to the observed decrease of the promoter activity, especially with respect to the *ndh* promoter. Altogether, downregulation of the respiratory gene promoters and the resulting decrease of the NADH oxidase activity indicate that *Z. mobilis* does not cope the ethanol stress by activation of its respiratory chain or any of its branches, but rather react to that condition by its partial shutdown.

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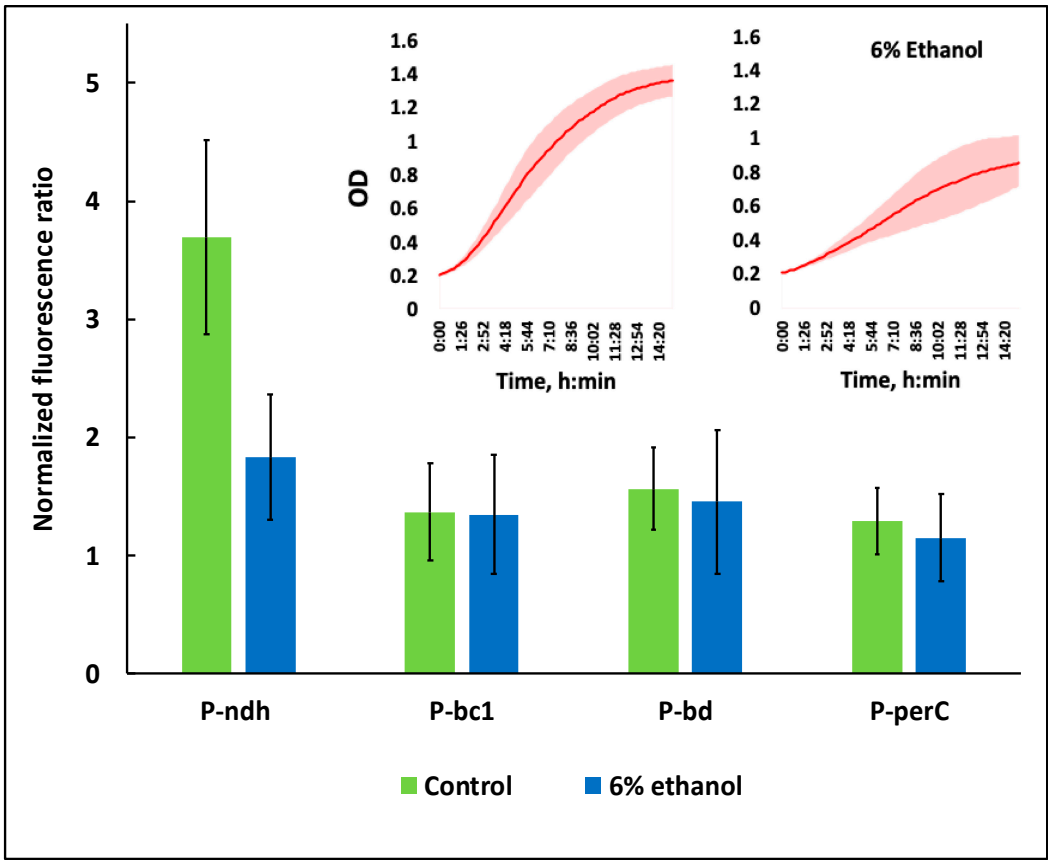

Figure 3. The effect of ethanol stress on the relative activity of respiratory gene promoters of strain Zm6. P-ndh, type II NADH:ubiquinone oxidoreductase promoter; P-bc1, promoter of cytochrome bc_1 operon; P-bd, promoter of cytochrome *bd* operon; P-perC, cytochrome *c* peroxidase promoter. Inset: the time course of batch growth of the strain Zm6_ pBBR-EGFP in the microplate reader under non-stress (left side curve) and ethanol stress (right side curve) condition. All presented values are means of 8 biological replicates (non-stress condition) or 5 biological replicates (stress condition). For details on calculation of the normalized fluorescence ratio, see the text.

4. Discussion

In the present study we show that the respiratory chain of *Zymomonas mobilis* has an important physiological function for keeping non-growing cells viable by driving oxidative phosphorylation with ethanol, the *Zymomonas* major catabolic product. Thus, *Z. mobilis* largely follows the MAC strategy of the Crabtree-positive yeast, implying under 'C' (consume) ethanol consumption specifically for maintenance of viability, but not for growth. Sugar-depleted media, exposed to air, and containing some amount of ethanol, probably represent a common niche in the natural life cycle of this bacterium. Previously, we have identified another advantage of respiration for survival of *Z. mobilis* in the same type of niche. As shown by Rutkis et al. [23], after glucose is added to a starving *Z. mobilis* cell suspension, the respiring strain takes it up more rapidly, than the Ndh-deficient mutant. Entner-Doudoroff pathway simulations indicate that it is the oxidation of extra NAD(P)H by the respiratory chain that accelerates the pathway flux, conferring a competitive advantage to the respiring strain, when limited amount of glucose suddenly becomes available. Furthermore, Jones-Burrage et al. [44] have found that on minimal growth medium under oxic condition the respiring wild-type strain ZM4 grows better than its Ndh-deficient mutant derivative – quite the opposite to what is seen on rich growth medium. In summary, survival of sugar deficiency and, perhaps, some other limitations in the growth medium is a distinct physiological domain where *Z. mobilis* makes use of its respiratory chain. The advantage provided by the respiratory chain for survival in sugar-depleted media might bear such a high adaptive priority for this bacterium that it cancels out the disadvantage for its aerobic growth on rich medium.

We also wanted to see, if the respiratory chain of *Z. mobilis* was involved in the protection of the cell under ethanol stress conditions. It has been demonstrated that various stressors in microorganisms induce a burst of the reactive oxygen species (ROS) production, leading to the secondary oxidative stress [45]. Elevated ROS production takes place also under ethanol stress, as reported e.g., for *E. coli* and *S. cerevisiae* [46,47]. Accordingly, respiratory chain that mitigates the ethanol stress might be expected to do so primarily by coping with the associated ROS production. Some evidence has accumulated on the role of *Z. mobilis* respiratory chain in the oxidative stress defense. Martien et al. [48] have reported upregulation of several respiratory chain components after exposing of a strictly anaerobic culture of *Z. mobilis* Zm4 to vigorous aeration. They have suggested that the cytochrome *c* peroxidase and cytochrome *bd* function to limit the toxic effects of oxygen. In support of that, the cytochrome *c* peroxidase-negative strains of *Z. mobilis* Zm6 and *Z. mobilis* TISTR 548 are shown to be hypersensitive to exogenously added hydrogen peroxide [21,49], pointing to some protective activity of the respiratory peroxidase branch (Fig.1).

However, the relation of respiratory chain to the intracellular ROS levels is fairly complex. Along with activities like cytochome *c* peroxidase that remove hydrogen peroxide, the electron transport itself is a major source of ROS [50]. At higher degree of reduction of its electron carriers, the ROS production in the respiratory chain tends to increase [51]. We hypothesize that the downregulation of the respiratory promoters and the decrease of the NADH oxidase activity that we see under ethanol stress, is aimed primarily at minimizing the respiratory ROS production. Since the *ndh* promoter is downregulated much more than the rest, the 'entrance gates' of the electron transport chain might become the main bottleneck of the electron transport flux, while the capacity of the terminal oxidase remains almost unaffected. That should result in a more oxidized steady-state of the respiratory electron carriers, and hence, in a lower rate of respiratory ROS generation. Such reasoning seems to be in line with recent findings of Felczak and TerAvest [52]. They reported that, while the decrease of respiration rate by deletion of *ndh* in the strain ZM4 stimulated aerobic growth, a similar decrease of respiration attained by deletion of cytochrome *bd* had an opposite effect. One might speculate that in the *ndh* mutant the steady state degree of reduction of the electron carriers (and accordingly, ROS production) is much lower, than in the terminal oxidase-deficient strain. That might contribute to the observed difference in their aerobic growth pattern. This hypothesis, of course, needs experimental verification by monitoring the redox state of electron carriers (primarily, that of ubiquinone) and measurement of the rate of respiratory ROS production.

Altogether, the physiology and regulation of *Zymomonas mobilis* respiration under stress conditions requires further research, as it might reveal novel, non-conventional functions of bacterial respiration. Better understanding of the alternative roles of respiratory chain is important both for rational metabolic engineering of this bacterium, as well as for the microbial biotechnology in general.

Author Contributions: Conceptualization, U.K. and I.S.; methodology, I.S., R.R. and M.G.; software, R.R. and K.S.; validation, M.B., E.R.P. and U.K.; formal analysis, U.K.; investigation, I.S., M.B., E.R.P., K.S. and Z.L.; resources, R.R.; data curation, I.S., M.G. and U.K.; writing—original draft preparation, M.B., E.R.P. and U.K.; writing—review and editing, U.K., M.G.; visualization, M.B., E.R.P.; supervision, U.K., I.S.; project administration, R.R.; funding acquisition, R.R. and U.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Latvian State Education Development Agency ERDF project No.1.1.1.1/19/A/097.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2023 doi:10.20944/preprints202309.1412.v1

Appendix A

Table A1. The amplified promoter regions of the *Z. mobilis* Zm6 respiratory chain components, the *egfp* sequence, and the respective primer sequences used in the study. Sequences of the structural gene ORFs are shown in blue. The primer binding regions are given in bold. Sequences, edited to introduce restriction sites, are underlined.

Gene/operon names, and sequence i.d.	Sequences of the amplified chromosomal regions, bearing the promoters of the respective genes/operons (5' → 3')	Forward and reverse primer sequences (5' → 3')
Cytochrome *c* peroxidase *perC* ZZ6_0192	→ ZZ6_0191 **CCGATGAATGGGTAAAATAAGACCTTTTCC**CTTTCTAAAATTTCAGAGGTGGCTTTATTC CTGTTAAAGCCACCTTTTATTTTATCTTTTCTTTATTCTTTCTCAGCTTTTCTGATTAA AAAATTTTGTAAATATAGCCAATATTTCCAATGAAATATCAGGTTAAAATATCTGTAAAG AACGAGAATTTTCTTATCACTTTAATGATAACTATTGGCATCATCAAAATCATCAAGAAT TAATCAAAATTGAAATGACAGTTATGCCTTTTACTCTAAAAGGGATGTGCTTTCTGTCTT GGAATAAGGCCATATTTTTGTTAATTCACAATAAATAAAAAGGTAAATCATCTTCAATAG GGAATCGGATACTATAAATTTATAGCCGACCATCCGATCATCTCTGGCGCTGGATTAAGA TAACGAGG**GGCAAGCACAGTTTTATGAATATCAAGGCT**TTATT → ZZ6_0192	**F primer** BamHI CCGATGAATGGGATCCATAAGACC TTTTCC **R primer** EcoRI AGCCTTGATATTCATGAATTCGTG CTTGCC
Cytochrome bc_1 (ubiquinol:cyt*c* oxidoreductase) operon: FeS cluster (Rieske) subunit ZZ6_0349; Cyt *b* subunit ZZ6_0348; Cyt c_1 subunit ZZ6_0347	→ ZZ6_0350 CTCGCCGCAACCGTC**TTGTTCTGGCTATGAGTCCTGAGCCTAGAT**TTCATGATAGTCAAT ATATTAAAAAATTATATATTGTAGTAATCTTTCTTAAAAAATCAGAATAATTGATTTAAA TATCATTAATTATGGCATTTTAATTATCATTTTTAATATTTTCATGGACAATGAAGAAAA CTTATTATGTTTTTATTGTAGTAAGCGTTGCAAAAAATATTGTAAGTAATTTACAGTAGA GTA**GGTTCGTCAAGGGATATGAAGAATAGCGAA**GCGAA → ZZ6_0349	**F primer** BamHI TTGTTCTGGCTATGGATCCTGAGC CTAGAT **R primer** EcoRI TTCGCTATTCTTCATGAATTCTGA CGAACC
Cytochrome *bd* operon: *cydA* ZZ6_1532; *cydB* ZZ6_1531	ZZ6_1533 ← TCAAG**GGTGGAAATGGTCAT**GTCGCTTCAACCTTTGATTCTATGAGCGAGGAAAGACTTA GGAGATATATCTAACAAAATTCGACCCGATCTGACAGCTATTATTAAAATGCAGAATAAG GTCGCGGGCTTTTTATCAGCTAACTCTAACAAAAAACAGAATCATTCGAAAAAGAATAAT AGAATACTCTATATTTAAATTATAAATTGATCCTTTTTCATATGGCTTTTTAATCACAAC CGCCCTAAAATCGGAAAATATCTGTTCCCTTGGGTGGTCTGTTATCAGAGATAGAAAATA ATTCGATTTCAAAAATCGTTATTTTCAATATTGAGACCTAAGCCCCTTTTCCCTTGTTCC TATCGTGACTTTCTTTTGAAATCGCCCCTATTCAACGAATTTAGGGGCAAGTTTTAAAAT AAAGCCCTTAATCGCTTCATCACTATTTTATTTAAAAAATGTTATTGATCTATAAAAAGA TACTATAACTACAGCAATATACTCAAAACAATCTGACATGGTCATATTAAATTTTAAAAT TTATCAAAAAAATAATTAAAATTAAAGAAATATAAAACGATAATTAAACCCAATTGAAGA TAAATAGACTGTAGATAGAGATTATTACCCCCGAGGATCAAGAAATTCTCTATTGACACA ACACCCGATGCTATCAATACACCGATAATATATAGGGGGCATTTTACAGATTCAGGAAGC TGATCCCTGAAGAAAAGCCCGTAGACCCTAATAATTCCTAATATTATC**GACGGAGCCATT TCT**ATGGTACCAGATGCGACCGC → ZZ6_1532	**F primer** BamHI GGTGGAAATGGATCCGTCGCTTCA ACCTTT **R primer** EcoRI CGCATCTGGTACCATGAATTCGGC TCCGTC
Type II NADH: ubiquionone oxidoreductase *ndh* ZZ6_0213	ZZ6_0214 ← TGATC**GGTTTGTTTTTCCAT**AACCCATATTGACGGGAAAAGAAGCCAAAGGTCAATCAGA GAAAGCGCGCTTTAAACAAAGCCCATGAGGATGATCAAAGACAGAGACCGGATAAGGGCA AAGATAGTTTCACAGTAAGCAATAAAAACTTTCACTCTGACTTATAATTTATGTGAAACA GATTTGAATGTTCTTCGTTAGTTACAGCAAAAGGTAATTGAGGCGGCTCGCAAACCACCA CGCCAAAGGGGGCTGGGTC**GCTTGGAAGAGAATAGAGGTTTCAAATGTC**GAAGAATGGTA → ZZ6_0213	**F primer** BamHI GGTTTGTGGATCCATAACCCATAT TGACGG **R primer** EcoRI GACATGAATTCCCTCTATTCTCTT CCAAGC
EGFP gene in plasmid pQE-EGFP *egfp*	→ *egfp* **ATACGGATCCACCGGTCGCCACCATGGTGA**GCAAGGGCGAGGAGCTGTTCACCGGGGTGG TGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCG AGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCA AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCA GCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCT ACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGG TGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGG AGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATA TCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCG AGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCC CCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCA ACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCG GCATGGAC**GAGCTGTACAAGTAAGGAGGTTCCCGCGGA**	**F primer** EcoRI ATACGGATCCACCGGTCGAATTCA TGGTGA **R primer** HindIII TCCGCGGGAAAGCTTTTACTTGTA CAGCTC

 doi:10.20944/preprints202309.1412.v1

Appendix B

Table B1. The FTIR spectroscopy data on the relative macromolecular composition (% of dry weight) of the wild type strain Zm6 biomass before and after the 6h aerobic incubation period. Mean values of 4 biological replicates are presented.

Incubation without substrate addition	Carbohydrates		Nucleic acids		Proteins		Lipids	
	0h	6h	0h	6h	0h	6h	0h	6h
Mean value	12.65	11.97	17.57	18.45	57.74	57.71	2.05	1.87
± SEM	± 0.47	± 0.39	± 0.35	± 0.61	± 0.61	± 0.27	± 0.24	± 0.17
P value	0.33		0.28		0.97		0.55	
Incubation with 1 g L^{-1} glucose additions, once per hour								
Mean value	12.70	12.21	15.93	15.48	59.13	60.06	2.24	2.25
± SEM	± 0.87	± 0.39	± 0.19	± 0.15	± 0.68	± 0.49	± 0.36	± 0.10
P value	0.63		0.11		0.31		0.98	

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