Engineering *Escherichia coli* for efficient conversion of glucose to pyruvate

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*Escherichia coli* TC44, a derivative of W3110, was engineered for the production of pyruvate from glucose by combining mutations to minimize ATP yield, cell growth, and CO₂ production (∆atpFH ∆adhE ∆sucA) with mutations that eliminate acetate production (poxB::FRT (FLP recognition target) ∆ackA) and fermentation products (∆focA- pflB ∆frdBC ∆ldhA ∆adhE). In mineral salts medium containing glucose as the sole carbon source, strain TC44(∆focA-pflB ∆frdBC ∆ldhA ∆atpFH ∆adhE ∆sucA poxB::FRT ∆ackA) converted glucose to pyruvate with a yield of 0.75 g of pyruvate per g of glucose (77.9% of theoretical yield; 1.2 g of pyruvate liters⁻¹hr⁻¹). A maximum of 749 mM pyruvate was produced with excess glucose. Glycolytic flux was >50% faster for TC44 producing pyruvate than for the wild-type W3110 during fully aerobic metabolism. The tolerance of *E. coli* to such drastic changes in metabolic flow and energy production implies considerable elasticity in permitted pool sizes for key metabolic intermediates such as pyruvate and acetyl-CoA. In strain TC44, pyruvate yield, pyruvate titer, and the rate of pyruvate production in mineral salts medium were equivalent or better than previously reported for other biocatalysts (yeast and bacteria) requiring complex vitamin feeding strategies and complex nutrients. TC44 offers the potential to improve the economics of pyruvate production by reducing the costs of materials, product purification, and waste disposal.

Materials and Methods

**Microorganisms and Media.** *E. coli* W3110 (ATCC 27325) derivatives and LY01 (25) (Table 1) were grown on mineral salts medium (26) containing glucose (2% in plates, 3% in broth). When needed for pH control, 3-[N-morpholino]propanesulfonic acid (0.1 M, pH 7.4) was added but was not included in pH-controlled fermentations. During plasmid and strain construction, cultures were grown in LB broth or on LB plates (1.5% agar) (27). Glucose (2%) was added to LB medium for all strains containing mutations in (*F₁:F₀*)⁺ ATP synthase. Antibiotics were included as appropriate (50 mg/liter kanamycin, 50 mg/liter ampicillin, 50 mg/liter apramycin, and 12.5 or 6.25 mg/liter tetracycline).

**Genetic Methods.** Standard methods were used for plasmid construction, phage P1 transduction, electroporation, and PCR (27, 28). Coding regions for *ackA* and *poxB* were amplified by using ORFmer primers (Sigma-Genosys, The Woodlands, TX) and initially cloned into pCR2.1-TOPO (Invitrogen). Chromosomal integration of mutated genes was facilitated by pKD46 containing an arabinose-inducible Red recombinase (29). Integrants were selected by using antibiotic resistance, screened for appropriate antibiotic resistance markers and phenotypic traits, and verified by analyses of PCR products and fermentation profiles. FRT (FLP recognition target)-flanked antibiotic resistance genes were deleted by using FLP recombinase (30, 31).

**Disruption of Pyruvate Oxidase (poxB).** A single derivative of pCR2.1-TOPO was selected in which the amplified *poxB* gene

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**Abbreviation:** FRT, FLP recognition target.

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was oriented in the same direction as the lac promoter (pLOI2075). To eliminate extraneous BsaI sites in the vector, the small EcoRI fragment containing poxB was ligated into the corresponding site of pLOI2403 to produce pLOI2078. The Smal fragment from pLOI2065 containing a tet gene flanked by FRT sites was ligated into the unique BsaI site in poxB to produce pLOI2080. Plasmid pLOI2080 served as a template for the amplification of poxB::FRT-tet-FRT (3.4 kb) with poxB primers. Amplified DNA was integrated into _E. coli_ W3110(pKD46) as described (26) to produce LY74. The poxB::FRT-tet-FRT mutation in LY74 was transduced into TC36 to produce TC41. The tet gene was removed by using FLP recombinate, and the resulting strain was designated TC42 (ΔpoxB::FRT ΔfrdBC::FRT ΔatpFH::FRT ΔatpFH::FRT Δadhe::FRT ΔsucA::FRT poxB::FRT).

**Deletion of ackA (Acetate Kinase).** The ΔackA::FRT-tet-FRT mutation was transduced from SZ61 (8) into TC36 and TC42 to produce strains TC37 (ΔpoxB::FRT ΔfrdBC::FRT ΔldhA ΔatpFH::FRT Δadhe::FRT ΔsucA::FRT ΔackA::FRT-tet-FRT) and TC43 (ΔpoxB::FRT ΔfrdBC::FRT ΔldhA ΔatpFH::FRT Δadhe::FRT ΔsucA::FRT poxB::FRT ΔackA::FRT-tet-FRT), respectively. After removal of the tet gene by using FLP recombinate, resulting strains were designated TC38 (ΔpoxB::FRT ΔfrdBC::FRT ΔldhA ΔatpFH::FRT Δadhe::FRT ΔsucA::FRT ΔackA::FRT-tet-FRT) and TC44 (ΔpoxB::FRT ΔfrdBC::FRT ΔldhA ΔatpFH::FRT Δadhe::FRT ΔsucA::FRT poxB::FRT ΔackA::FRT-tet-FRT), respectively.

**Fermentation.** Fermentations (5 and 10 liters) were conducted at 37°C (dual Rushton impellers, 350–450 rpm; pH 7.0) in BioFlo 3000 fermentors (New Brunswick Scientific). Unless stated otherwise, dissolved oxygen levels were 100% of air saturation at the time of inoculation and allowed to fall to 5% of air saturation during cell growth with continuous air sparging (0.2 vvm (vessel volume per minute)). This level of oxygen was maintained by mixing O2 with air at a constant flow rate of 1.0 liter/min. Broth pH was controlled with 11.4 M KOH. For fed-batch studies, glucose was added from a sterile 4-M stock. Two fed batch regimes were investigated: (i) 3% initial glucose followed by the addition of 3% glucose after 15 h (6% total glucose) and (ii) 3% initial glucose with the addition of 590 ml of 4 M glucose at a constant rate over a 20-h period (9.8% total glucose).

Seed cultures prepared as described (26) were used to provide an inoculum of 16.5 mg of dry cell weight per liter. Broth samples were removed to measure organic acids, residual glucose, and cell mass. Volumetric and specific rates were estimated from measured values for glucose and acetate by using PRISM (GraphPad, San Diego). A smooth curve was generated with 10 points per min (Lowess method) to fit measured results. The first derivative (acetate or glucose versus time) of each curve served as an estimate of volumetric rate. Specific rates (mM liter−1 h−1 mg−1 dry cell weight) were calculated by dividing volumetric rates by respective values for cell mass.

**Analyses.** Organic acids and glucose were measured with a Hewlett-Packard HPLC (HP 1090 series II) equipped with a UV monitor (210 nm) and refractive index detector (26). Cell mass was estimated with a Bausch & Lomb Spectronic 70 spectrophotometer (1.0 OD580 nm is equivalent to 0.33 g/liter dry cell weight).

**Results.** Pyruvate _As a Coproduct During Acetate Fermentations._ _E. coli_ TC36 (ΔpoxB::FRT ΔfrdBC ΔldhA ΔatpFH Δadhe ΔsucA ΔackA ΔfocA-pflB) was previously engineered from W3110 (wild type) for the production of acetate (Fig. 1A) by combining chromosomal deletions that minimize cell yield, oxygen consumption, CO2 evolution, and reduced fermentation products (26). In this strain, glycolytic flux was higher than the parent because of a deletion of genes (ΔatpFH) encoding two membrane proteins that couple the F1 and F0 components of the (F1F0)H+-ATP synthase complex. This mutation eliminated ATP production by oxidative phosphorylation and created an active, cytoplasmic F1-ATPase (Fig. 1B and C). Glycolytic flux in TC36 exceeded the capacity for acetate production under our initial test conditions (5% air saturation at inoculation and during fermentation), resulting in the transient accumulation of ~16 mM pyruvate near the end of exponential growth (Fig. 2). The peak level of pyruvate was increased to 81 mM (Fig. 2) by inoculating the fermentor at an initial dissolved oxygen level of 100% air saturation (rather than 5% of saturation) with continuous air sparging until the oxygen level declined to 5% air saturation, then adding oxygen to maintain 5% of air saturation. Under this condition, pyruvate yield at the peak was 25% of the maximum theoretical yield (Fig. 2).

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**Table 1. Sources and characteristics of strains and plasmids**

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Relevant characteristics</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>W3110</td>
<td>K12 wild type</td>
<td>ATCC 27235</td>
</tr>
<tr>
<td>TOP10F</td>
<td>lacP (episome)</td>
<td>Invitrogen 25</td>
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<tr>
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<td>E. coli B, fed p; δdeo, adhEcat</td>
<td>This study 8</td>
</tr>
<tr>
<td>LY74</td>
<td>W3110, ΔpoxB::FRT-tet-FRT</td>
<td>This study</td>
</tr>
<tr>
<td>S261</td>
<td>W3110, ΔackA::FRT-tet-FRT</td>
<td>This study</td>
</tr>
<tr>
<td>TC36</td>
<td>W3110, (Suc’), ΔfocA-pflB::FRT ΔfrdBC ΔldhA ΔatpFH::FRT ΔatpFH::FRT Δadhe::FRT ΔsucA::FRT</td>
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</tr>
<tr>
<td>TC37</td>
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</tr>
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</tr>
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<tr>
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<td>This study</td>
</tr>
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<td>This study</td>
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<tr>
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<td>pCR2.1-TOPO</td>
<td>Invitrogen 27</td>
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</tr>
<tr>
<td>Bla kan tet FRT</td>
<td>pLO12043</td>
<td>29</td>
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We have explored additional genetic modifications of TC36 to further increase the efficiency of pyruvate production from glucose. Although many metabolic routes lead to acetate, two primary routes are present in *E. coli* (Fig. 1A): (i) conversion of acetyl-CoA to acetate by phosphotransacetylase (*pta*) and acetate kinase (*ackA*) and (ii) direct oxidation of pyruvate to acetate by pyruvate oxidase (*poxB*) (Fig. 1A). Derivatives of TC36 were constructed with mutations in both pathways.

**Acetate Kinase (ΔackA) Enhances Pyruvate Production but Inhibits Glycolysis.** To block acetate production by the acetate kinase pathway, the central region of the *ackA* gene was deleted in TC36 to produce TC38. Acetate production was reduced by 85% (Fig. 3C and Table 2), consistent with the acetate kinase pathway being the dominant route for acetate production in TC36. However, this deletion also reduced net ATP production (estimated) by 30% (Fig. 1A), reduced cell yield by 36% (Fig. 3A and Table 2), and reduced the maximum specific growth rate by 45% (Table 3). Glycolytic flux was reduced by 45% (Table 3), increasing the time required to complete fermentations from 18 h for TC36 to 24 h for TC38 (Fig. 3B). Although both volumetric and specific rates of glucose metabolism were lower for TC38 (Table 3), pyruvate yield was 5.5-fold higher (Table 2 and Fig. 3D), and the specific rate of pyruvate production was 4-fold higher (Table 3) than for TC36. Small amounts of 2-oxoglutarate, succinate, and fumarate were produced by both strains. Ten percent to 15% of the carbon was not recovered as cell mass or acidic fermentation products and is presumed to be lost as CO2 because of metabolite cycling. With strain TC38, the pyruvate yield (195 mM) was 58% of the theoretical maximum. Acetate (28.9 mM) remained the second most abundant product.

**Pyruvate Oxidase (poxB) Mutation Causes a Small Reduction in Acetate Production.** Pyruvate can be metabolized to acetate by the membrane-bound protein pyruvate oxidase by using the electron transport system to couple oxygen as a terminal electron acceptor. The *poxB* gene is typically repressed during exponential growth but is induced by stress or entry into stationary phase (32, 33). The *poxB* gene in TC36 was disrupted by constructing stop codons in the central region to produce TC42. In contrast to the *ackA* deletion (TC38), the *poxB* mutation (TC42) caused a relatively small decrease in acetate and an increase in pyruvate (Table 2 and Fig. 3C and D), consistent with a minor role for the PoxB pathway. Pyruvate yield with TC42 was <30% of the theoretical maximum. Unlike the mutation in *ackA* (TC38), inactivation of *poxB* (TC42) did not reduce the rate of growth or glucose metabolism (Table 3).

**Combining Mutations in Pyruvate Oxidase (poxB) and Acetate Kinase (ackA) Increased Pyruvate Production.** Strain TC44 (ΔoxA-gfB ΔsfdBC ΔldhA ΔtpFH ΔadhE Δ sucA poxB::FRT ΔackA) was constructed in which both the acetate kinase and pyruvate oxidase mutations were combined in a TC36 background. Inactivation of *poxB* was beneficial for growth and pyruvate production (Fig. 3A).
and Tables 2 and 3) in comparison to TC38 (an isogenic strain containing functional poxB). Both volumetric and specific rates of glucose metabolism were higher for TC44 than for TC36 or TC38 (Table 3). Acetate production by TC44 was half that of TC38, and the pyruvate yield was 17% higher. The specific rate of pyruvate production by TC44 was 8-fold that of TC36 and twice that of TC38 (Table 3). The time required to complete fermentation with TC44 was 30% shorter than with TC38 (Fig. 3B). Broth containing 3% glucose (167 mM) was metabolized to 2.2% pyruvate (252 mM) after 18 h in mineral salts medium (Fig. 3D). Although acetate levels were substantially reduced by combining the poxB and ackA mutations (Fig. 3C), acetate (12 mM) and dicarboxylic acids (20 mM) remained as minor products.

**The Beneficial Role of a poxB Mutation for Pyruvate Production.** Although eliminating the primary route for acetyl-CoA dissimilation (ackA) in TC38 increased pyruvate production, this was accompanied by detrimental effects on growth and fermentation rates (Fig. 2 and Tables 2 and 3) that were substantially reduced by inactivation of PoxB (TC44). Pyruvate oxidase competes with
cytoplasmic F1-ATPase, increasing the availability of ADP for the elimination of all major nonessential pathways (Table 4). Typical strains, such as T. glabrata no. 8, a yeast strain currently used for the commercial production of pyruvate, can currently be grown with 749 mM pyruvate. This may represent the total pool size required for glycolysis and minimizing allosteric repression by ATP accumulation. A similarly high flux was previously reported for an analogous strain designed for the efficient production of acetate (26). Strain TC44 converted glucose to pyruvate with a yield of 0.75 g of pyruvate per g of glucose (77.9% of theoretical yield) under optimal conditions in minimal medium. With excess glucose, a maximum of 749 mM pyruvate was produced. Addition of a pyruvate oxidase mutation was surprisingly beneficial for growth and pyruvate production. Introduction of the poxB deletion into the acetate-producing strain (TC36) resulted in a small improvement in growth rate, in contrast to the ackA deletion that reduced growth rate by half (Table 3). The decreased growth rate in the acetate kinase mutant of TC36 cannot be explained by the predicted 30% reduction in ATP yield, because the growth rate was substantially restored by adding a subsequent mutation in pyruvate oxidase, which should not affect ATP production. These results suggested that the reduction in growth rate by the acetate kinase mutation may result from an increase in PoxB activity associated with a larger pyruvate pool. Pyruvate oxidase transfers electrons from pyruvate to ubiquinone on the decarboxylation of pyruvate. An increase in pyruvate metabolized by pyruvate oxidase would increase oxygen demand and reduce the oxygen available for NADH oxidation (37). Because the total nicotinamide adenine dinucleotide pool is relatively constant (38) and the NAD+/NADH ratio is responsive to changes in oxygen availability (38), increased pyruvate oxidase activity would also reduce the level of NAD+ available for glycolysis. High NADH levels also serve as an important allosteric regulator of key enzymes such as citrate synthase (35) and phosphoenolpyruvate carboxylase (36). Allosteric inhibition of these enzymes in minimal medium would limit both glycolytic flux and the partitioning of carbon into biosynthesis. Oxidation of NADH is also essential to provide the stoichiometric levels of NAD+ required for glycolysis. Thus, inactivating pyruvate oxidase may be expected to improve growth by eliminating the increase in oxygen demand, restoring the NAD+ pools for glycolysis, and decreasing the level of NADH. This hypothesis was supported by further tests in which the availability of oxygen was increased from 5% of air saturation to 50% of air saturation during fermentation with the acetate kinase mutant TC38. The increase in oxygen (50% air saturation) substantially reversed the negative impact of the acetate kinase mutation on growth rate and metabolism, analogous to the effect of adding a pyruvate oxidase mutation. A similar increase in oxygen was of no benefit for TC44 in which both acetate pathways had been mutated (data not shown).

Discussion

Construction of E. coli TC44 for pyruvate excretion during the oxidative metabolism of glucose required mutations to reduce the utilization of pyruvate for cell growth (ΔatpFH ΔsucA) and the elimination of all major nonessential pathways (ΔfocA-pflB ΔfrdBC ΔldhA adhE poxB ΔFRT ΔackA) that can consume pyruvate. In this strain, glycolytic flux exceeded that of the unmodified parent, W3110, by >50%. This increased rate of glycolysis is attributed to the ATPase (ΔatpFH) mutation that provided a cytoplasmic F1-ATPase, increasing the availability of ADP for glycolysis and minimizing allosteric inhibition by ATP accumulation. A similarly high flux was previously reported for an analogous strain designed for the efficient production of acetate.

Table 3. Comparison of metabolic rates

<table>
<thead>
<tr>
<th>Strain</th>
<th>μmax, g dry cell weight h⁻¹</th>
<th>Glucose consumption rate</th>
<th>Pyruvate production rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Volumetric, * g liter⁻¹ h⁻¹</td>
<td>Specific, † g liter⁻¹ h⁻¹ dcw</td>
</tr>
<tr>
<td>E. coli W3110 (wild type)</td>
<td>0.69</td>
<td>11.9</td>
<td>17.35</td>
</tr>
<tr>
<td>TC36 (ΔfocA-pflB frdBC ldhA atpFH adhE sucA)</td>
<td>0.49 ± 0.03</td>
<td>10.1 ± 2.6</td>
<td>17.6 ± 1.5</td>
</tr>
<tr>
<td>TC38 (ΔfocA-pflB frdBC ldhA atpFH adhE sucA)</td>
<td>0.51 ± 0.01</td>
<td>10.7 ± 0.9</td>
<td>29.7 ± 3.5</td>
</tr>
<tr>
<td>TC39 (ΔfocA-pflB frdBC ldhA atpFH adhE sucA ackA)</td>
<td>0.28 ± 0.01</td>
<td>6.7 ± 0.6</td>
<td>16.3 ± 2.2</td>
</tr>
<tr>
<td>TC42 (ΔfocA-pflB frdBC ldhA atpFH adhE sucA poxB)</td>
<td>0.21</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td>TC44 (ΔfocA-pflB frdBC ldhA atpFH adhE sucA poxB ackA)</td>
<td>0.34 ± 0.02</td>
<td>9.7 ± 0.7</td>
<td>27.2 ± 4.1</td>
</tr>
</tbody>
</table>

*Standard deviations are included for data from three or more fermentations; others represent an average of two fermentations.
†Maximum specific rates per g of dry cell weight (dcw).
achieve pyruvate titers of 69 g/liter, although yields are somewhat lower than for TC44 and more elaborate process controls are required. T. glabara strains used in the commercial process are multivitamin auxotrophs requiring tight regulation of vitamin concentrations that result in complex vitamin feeding strategies during fermentation (16). Previous E. coli strains constructed for pyruvate production were cultured in complex media and achieved low titers (22, 23). In contrast, E. coli TC44 produced pyruvate at high yields and high titers when grown with only minimal salts, glucose, and simple process controls.

The remarkable tolerance of E. coli to drastic changes in metabolic fluxes that allow the production of acetate or pyruvate as alternative products of metabolism implies considerable elasticity in permitted pool sizes for key metabolic intermediates, such as pyruvate and acetyl-CoA. It is interesting to note that pyruvate transiently accumulated in broth during fermentations with TC36 but was rapidly cometabolized with glucose during the latter stages of fermentation. Similar transient accumulation and reutilization was previously observed in minimal medium for other central metabolites (carboxylic acids and acetate) in other derivatives of W3110 (26). Exogenously added pyruvate has been shown to be rapidly mixed and metabolized with intracellular pyruvate (39). Carboxylic acids (40, 41) and acetate (5, 26) are also cometabolized with sugars. Active transport systems have been identified in E. coli for pyruvate (42), acetate (43), and dicarboxylic acids (44–46). Thus, for pyruvate and perhaps many other compounds, the extracellular milieu may be reasonably regarded as a reservoir for the temporary expansion of metabolic pools. A similar argument can be made for CO2 (47) and for acetaldehyde during ethanol production (48–50). The transient accumulation of metabolites in the aqueous milieu may be of evolutionary advantage. This accumulation could arguably be regarded as a form of metabolic conditioning of the environment, as a storage reservoir, and as an expanded metabolic pool to relieve a temporary imbalance in metabolic flux. Enzymes are relatively stable in E. coli. Substantial remodeling of metabolism is primarily regulated at the level of transcription with dilution during subsequent growth. Transient storage of products from imbalanced metabolism in the extracellular milieu could increase the metabolic flexibility of E. coli during adaptation to a changing environment.

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### Table 4. Comparison of biocatalysts for pyruvate production

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype/phenotype</th>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>Fermentation time, h</th>
<th>[Pyruvate], g/liter</th>
<th>Volumetric production, g/liter h(^{-1})</th>
<th>Pyruvate yield, g/g</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida lipolytica AJ 14352</td>
<td>B/3 Met-</td>
<td>Glucose</td>
<td>NH4NO3</td>
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<td>44</td>
<td>0.61</td>
<td>0.44</td>
<td>16</td>
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<tr>
<td>Debaryomyces hansenii Y-256</td>
<td>B/2 Bio-</td>
<td>Glucose</td>
<td>Peptone</td>
<td>96</td>
<td>42</td>
<td>0.44</td>
<td>0.42</td>
<td>16</td>
</tr>
<tr>
<td>T. glabara ACII-3</td>
<td>B/1 Bio- B/2 NA acetate leaky</td>
<td>Glucose</td>
<td>Soy hydrolase (NH4)2SO4</td>
<td>47</td>
<td>60</td>
<td>1.28</td>
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<td>16</td>
</tr>
<tr>
<td>T. glabara WSH-IP 303</td>
<td>B/1 Bio- B/2 NA</td>
<td>Glucose</td>
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<td>56</td>
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<td>(NH4)2HPO4</td>
<td>43</td>
<td>52</td>
<td>1.21</td>
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