INTRODUCTION

*Rhizopus oryzae* (R. oryzae) is a filamentous fungus belonging to the class of zygomycetes. It is able to ferment a wide range of carbohydrates, hydrolyzed from lignocellulosic materials and even cellobiase, to produce ethanol. However, *R. oryzae* also produces lactic acid as a major metabolite, which reduces the yield of ethanol.

**OBJECTIVE**

This study was conducted to determine whether; (i) using direct delivery of short (25 nt) synthetic siRNA can suppress the *ldhA* gene and make a significant reduction of lactic acid production in *R. oryzae*, and (ii) silencing of *ldhA* gene can alter the ethanol production, and favorably increase the yield of ethanol production in the fermentation process considering the biochemical pathway of ethanol production in *R. oryzae*.

**METHOD**

**Fungal strain**

The fungus *Rhizopus oryzae* CCUG 28958 (Culture Collection, University of Gothenburg, Sweden) was used in all experiments.

**siRNA**

In order to design the siRNA, the part of the gene with the lowest homology with other genes and most homology with responsible *ldh* genes was selected. siRNA was provided from Invitrogen, Carlsbad, CA.

**Fungal protoplast formation and transformation**

protoplasts were prepared by adding digestion buffer consists of Lysozyme (Sigma-Aldrich) to the mycelia. The CaCl2/PEG (polyethylene glycol) method was used to transform *R. oryzae* with siRNA.

**Batch culture fermentation**

In order to examine the efficiency of knockdown, a fermentation process was carried out with both wild type and knockdown *R. oryzae*. The cultivations were done in Erlenmeyer flask containing semi-synthetic growth media, which were inoculated with spore solution and cultivated to examine the growth of the cells.

**Analytical method – HPLC**

The concentrations of glucose, ethanol, lactic acid, and some other metabolites were examined using High Performance Liquid Chromatography (HPLC). A hydrogen-based ion-exchange column was used.

RESULTS AND DISCUSSION

**Designing an efficient siRNA**

Among two different genes (*ldhA* and *ldhB*), coding for NAD+ dependent L-lactate dehydrogenases in *R. oryzae*, *ldhA* gene is the main responsible gene. According to the sequence alignments *ldhA* and *ldhB* genes are similar to each other, exhibiting more than 89% nucleotide sequence identity (Figure 1). siRNA was designed in such a way that affects both *ldhA* and *ldhB* mRNAs.

**Effect of *ldhA* gene silencing on lactic acid and ethanol production**

The results represent 85.7% reduction in lactic acid production. These may be interpreted to show that silencing of lactic acid gene has been successful. Furthermore, the ethanol production increased 15.4% after RNA silencing of the lactate dehydrogenase gene (Table 1 and Figure 2).

**Effect of RNA silencing on other metabolites of glucose metabolism pathway**

Suppression of lactic acid production in the glucose metabolism pathway triggers pyruvate to mainly go through the production of other metabolites. After RNA silencing of lactate dehydrogenase gene, the yields of glycerol, succinic acid and pyruvate were affected too and the biomass concentration was decreased (Figure 3).

**Table 1: Product yield from anaerobic batch fermentation**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Lactic acid (mg/g)</th>
<th>Ethanol (mg/g)</th>
<th>Glycerol (mg/g)</th>
<th>Succinic acid (mg/g)</th>
<th>Pyruvate (mg/g)</th>
</tr>
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<tbody>
<tr>
<td>#1</td>
<td>3,7</td>
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<td>72</td>
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<td>3,3</td>
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<tr>
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<td>9,8</td>
<td>447</td>
<td>72</td>
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<td>447</td>
<td>73</td>
<td>0,2</td>
<td>2,9</td>
</tr>
</tbody>
</table>

**Wilcoxon rank sum test**

The samples were tested by using the R statistical program. Wilcoxon rank sum test with statistical significance on the 5% level. The p-values showed that the silenced *ldhA* gene in *R. oryzae* could reduce the lactic acid production (p-value=0.033) and increase the ethanol production (p-value=0.036) in knockdowns compared with the wild types.

**FUNDING SOURCE**

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