Abstract:

Title: IMPROVEMENT OF ETHANOL YIELD AND REDUCTION OF BIOMASS ACCUMULATION IN THE RECOMBINANT STRAIN OF SACCHAROMYCES CEREVISIAE OVEREXpressing ATP DEGRADING THE ENZYMES

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(54) Title: IMPROVEMENT OF ETHANOL YIELD AND REDUCTION OF BIOMASS ACCUMULATION IN THE RECOMBINANT STRAIN OF SACCHAROMYCES CEREVISIAE OVEREXpressing ATP DEGRADING THE ENZYMES

(57) Abstract: A new approach for increase of ethanol yield during alcoholic fermentation via decrease of biomass accumulation by using ATP degrading enzymes is described. The part of the Saccharomyces cerevisiae Ssbi gene coding for cytosolic ATPase domain of ribosome associated chaperon cloned into expression cassette under control of the glycerol-3-phosphate dehydrogenase gene (GPD1) promoter was introduced into the S. cerevisiae BY4742 strain. The recombinant strains were tested for their ability to grow and produce ethanol during glucose anaerobic and aerobic cultivations. Strains overexpressing ATPase domain of Ssbi possessed decreased concentration of intracellular ATP. They accumulated elevated amounts of ethanol and were characterized by decreased biomass accumulation as compared to the wild-type strain under both anaerobic and aerobic conditions. Similarly, the apyrase gene apy from E. coH encoding ATP/ADP hydrolyzing phosphatase and ATPase domain of Ssbi gene of S. cerevisiae were co-expressed under the control of galactose-inducible GALI promoter. The recombinant S. cerevisiae strains revealed slight reduction of biomass accumulation, while specific ATPase activity, ethanol accumulation and yield during alcoholic galactose fermentation under semi anaerobic conditions were increased.
INTERNATIONAL SEARCH REPORT

International application No
PCT/US 10/40167

A CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 1/00; C12N 9/14; C12P 7/06 (201 001)
USPC - 435/255.2; 435/195; 435/1 61;
According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC(8)-C12N 1/00, C12N 9/14, C12P 7/06 (2010 01)
USPC- 435/255 2, 435/195, 435/161

Minimum documentation searched to the extent that such documents are included in the fields searched
USPC- 435/254 1 1, 435/183, 435/252 3, 435/254 1, 435/320 1, 435/4 1, 435/440, 435/6, 435/69 1, 435/71 1, 435/483, 536/23 2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 435/254 2, 435/183, 435/252 3, 435/254 1, 435/320 1, 435/4 1, 435/440, 435/6, 435/69 1, 435/71 1, 435/483, 536/23 2

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB), Google Patents, Google Scholar fermentation, atp, hydrolysis, ethanol, alcohol, alpase, apyrase, anaerobic, yeast, production, increased, enhanced
GenCore 6 3 SEQ ID NO 2-3

C DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>X <strong>T</strong></td>
<td>US 2006/0949078 A1 (JESEN et al) 04 May 2006 (04 05 2006) para [0007]-[0010], [0046]-[0048], [0009], [0001], [0081]</td>
<td>1-2 and 13-18</td>
</tr>
</tbody>
</table>

D Further documents are listed in the continuation of Box C

* Special categories of cited documents
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
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Date of the actual completion of the international search
13 December 2010 (13 12 2010)

Date of mailing of the international search report
27 DEC 2010

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Form PCT/ISA/210 (second sheet) (July 2009)
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **Claims Nos** because they relate to subject matter not required to be searched by this Authority, namely

2. **Claims Nos** because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically

3. **Claims Nos** because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 64(a)

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**Box No. III** Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Groups I-III claims 1-24, drawn to a yeast strain comprising a recombinant nucleic acid that encodes an ATP degrading protein that lowers ATP levels in the cytosol of the yeast characterized in that the recombinant nucleic acid includes a promoter operable operably linked to overexpress a nucleic acid encoding at least one enzyme, and a method of using said strain. The first invention is restricted to cytosol soluble ATPase SSB2, to an apyrase, and to a combination of soluble ATPase and an apyrase, respectively. The exact claims searched will depend on the specifically elected enzyme(s) or a combination thereof. (NOTE claims 6-12 and 22-24 were excluded from Group I because they pertain to a non-elected subject matter (apyrase expression).)

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**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation
- No protest accompanied the payment of additional search fees
The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

The inventions of Groups I-III share the technical feature of a yeast strain comprising a recombinant nucleic acid that encodes an ATP-degrading protein that lowers ATP levels in the cytosol of the yeast characterized in that the recombinant nucleic includes a promoter operably operably linked to overexpress a nucleic acid encoding at least one enzyme. However, this shared technical feature does not represent a contribution over prior art. Specifically, US 2006/0094078 A1 to Jensen et al. (hereinafter "Jensen") discloses a yeast strain comprising a recombinant nucleic acid that encodes an ATP-degrading protein that lowers ATP levels in the cytosol of the yeast (Example 6, "expression of a Truncated F-1 ATPase beta subunit from Phaffia rhodozyma in Saccharomyces cerevisiae" (title)), wherein "this truncated beta subunit that was encoded on pATPbeta included the region of the beta subunit which is thought to encode the catalytic site for ATP hydrolysis. The truncation of the N-terminal part of the beta subunit probably means that the protein will no longer be exported into the mitochondrion, but should stay within the yeast cytoplasm" (para [0072]). Furthermore, Jensen discloses that said plasmid pATPbeta A "gave rise to an ade + phenotype in the Saccharomyces cerevisiae strain, W301, which carries a mutation in the ADE2 gene" (para [0070]). The ade + phenotype results in increase of "ATP hydrolysis in the cytoplasm, thereby effecting the concentrations of adenine nucleotides in the cytoplasm" (para [0071]). Finally, a strain carrying said pATPbeta A plasmid was "converted into Rho strains (petit mutants, defective in oxidative phosphorylation) by standard treatment with ethidium bromide. The induction with galactose caused even stronger inhibition of growth in the Rho background, which further indicates that the cause of the growth inhibition is uncoupled ATP hydrolysis in the cytoplasm" (para [0074]), characterized in that the recombinant nucleic includes a promoter operably linked to overexpress a nucleic acid encoding a cytosol soluble ATPase (para [0007]; [0008]; [0046]; [0048]). As said yeast strain was known at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Another technical feature of the inventions listed as Groups I-III is the specific enzyme(s) recited therein. The inventions do not share a special technical feature, because 1) no significant structural similarities can readily be ascertained among the enzymes, and 2) cytosol soluble ATPase ATPase SSB2 was known in the art at the time of the invention, as evidenced by US 2003/0233675 A1 to Cao et al. (hereinafter "Cao") that discloses overexpression (para [0006]) of a S. cerevisiae SSB2 protein (para [0007], SEQ ID NO 1544) to produce transgenic plants with improved properties (para [0006]). Without a shared special technical feature, the inventions lack unity with one another.

Groups I-III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.