



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/00	A2	(11) International Publication Number: WO 99/24558 (43) International Publication Date: 20 May 1999 (20.05.99)
(21) International Application Number: PCT/EP98/07011 (22) International Filing Date: 30 October 1998 (30.10.98) (30) Priority Data: 97203370.8 30 October 1997 (30.10.97) EP (71) Applicant (for all designated States except US): MOGEN INTERNATIONAL N.V. [NL/NL]; Einsteinweg 97, NL-2333 CB Leiden (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): GODDIJN, Oscar, Johannes, Maria [NL/NL]; Oude Herengracht 5, NL-2312 LN Leiden (NL). PEN, Jan [NL/NL]; Heivlinder 19, NL-2317 JS Leiden (NL). (74) Agent: VAN WEZENBEEK, L., A., C., M.; Mogen International N.V., P.O. Box 628, NL-2300 AP Leiden (NL).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i> <i>With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.</i>
(54) Title: NOVEL HIGH-FERMENTING MICROORGANISMS (57) Abstract This invention describes novel microorganisms with an increased carbohydrate metabolism and/or fermentative capacity caused by the expression of a heterologous enzyme coding for trehalose phosphate synthase (TPS).		

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NOVEL HIGH-FERMENTING MICROORGANISMS

FIELD OF THE INVENTION

5 The present invention relates to improvement of fermenting capacity of microorganisms, specifically yeast and more specifically *Saccharomyces*.

BACKGROUND ART

10 Yeast strains for example belonging to the genus *Saccharomyces* are used worldwide in the production of ethanol, both as endproduct and for brewing purposes, and leavening of bread. Such yeasts are capable of fermenting sugars to approximately equimolar amounts of carbondioxide (CO₂) and ethanol under anaerobic conditions. Baker's yeast (*Saccharomyces cerevisiae*) is commercially available as cream yeast (15%-21% dry matter), compressed yeast (26%-33% dry matter),
15 active dry yeast (92%-94% dry matter) or instant dry yeast (94%-97% dry matter). The past decades, one of the most important goals in yeast research has been the improvement of the fermentative capacity of baker's yeast, resulting in improved CO₂-production rates. For this
20 purpose, both classical hybridisation and molecular genetic techniques have been used.

 One of the targets of the research has been the carbohydrate metabolism in yeast. It has appeared that hexokinase functions as a
25 signal molecule in the carbohydrate metabolism in the sense that a slower rate of hexose phosphorylation also reduces the first steps in the glycolysis (Hohmann, S. et al., Curr. Genet. 23, 281-289, 1993; van Dam, K. et al., Ant.V.Leeuwenh. 63, 315-321, 1993). This research has been done with yeast defective in trehalose metabolism (ggs1-
30 mutant, described in Thevelein, J.M., Ant.V.Leeuwenh. 62, 109-130, 1992). It appeared that the defective gene was TPS1, coding for trehalose-6-phosphate synthase (Bell, W. et al., Eur. J. Biochem. 209, 951-959, 1992). On basis of this research three possible models which may account for the interaction between a hexose transporter, hexose
35 phosphorylating enzyme and the trehalose synthesizing complex have been proposed (Thevelein, J.M., Hohmann, S., TIBS 20, 3-10, 1995). Each of the models is able to explain some observations, none of the models, however, is definitely established nor supported by

experimental evidence. It has been shown (EP 577 915) that *ggs1*-mutants (*ggs1-1*) show higher invertase and alpha-glucosidase activity and hence suppress the limiting step in yeast fermentation processes. However, the mechanism of action has not been unraveled.

5 There still remains a need for means of regulating carbohydrate metabolism, especially in yeast, resulting in strains having a high capacity of fermentation. Furthermore, elucidation of the controlling mechanism on glycolysis and other metabolic processes is an object of ongoing research.

SUMMARY OF THE INVENTION

10 It has now been found that microorganisms having an increased fermentation capacity can be obtained by providing them with a recombinant DNA capable of expressing TPS, this recombinant DNA preferably being of heterologous origin, preferably selected from the group of bacterial, fungal, plant, animal and human DNA, more preferably from *Escherichia coli*.

15 More specifically the microorganism is a yeast, preferably a yeast of a strain of *Saccharomyces*, more preferably *Saccharomyces cerevisiae*.

20 Another object of the invention is to provide a microorganism having an altered carbohydrate metabolism and/or fermentation capacity characterized in that this alteration is caused by a recombinant DNA expressing a protein which influences the endogenous level of trehalose-6-phosphate.

25 Also object of the invention is a method for modifying the carbohydrate metabolism of a microorganism and/or the fermentation capacity of said microorganism by providing it with a recombinant DNA expressing TPS. The recombinant DNA in this method will preferably be a heterologous DNA sequence, preferably selected from the group of bacterial, fungal, plant, animal and human DNA, more preferably from *Escherichia coli*.

30 Further object of the invention are microorganisms having a modified carbohydrate metabolism and/or fermentation capacity characterized in that this alteration is caused by a recombinant DNA expressing a product which influences the endogenous level of trehalose-6-phosphate, the product preferably selected from the group consisting of TPS, TPP, trehalase, trehalose phosphorylase, trehalose hydrolase and anti-sense trehalase.

Specifically the invention describes a method for providing microorganisms having an increased fermentation capacity by being provided with a recombinant DNA capable of expressing TPS, this recombinant DNA preferably being of heterologous origin, preferably selected from the group of bacterial, fungal, plant, animal and human DNA, more preferably from *Escherichia coli*.

More specifically the microorganism used in this method is a yeast, preferably a yeast of a strain of *Saccharomyces*, more preferably *Saccharomyces cerevisiae*.

Another object of the invention is improved dough for use in bakery, comprising a yeast according to this invention. Also comprised is a method for baking using said dough, and bread or other bakery products baked by this method.

A further object of the invention is a method for ethanol production with the microorganisms of the invention. Also a method for beer brewing or brewing other alcoholic beverages forms part of the invention. Accordingly, also the beverages produced by such a method are comprised in this invention.

DESCRIPTION OF THE FIGURES

Figure 1: Measurement of the fermentation capacity of a reference strain (Mog2) and an isogenic strain which contains a cassette expressing TPS (Mog4). Fermentation capacity is measured by ethanol production versus time in an anaerobic reaction vessel under CO₂. Concentration of biomass was 2.00 g/l. The figure shows the results of a single comparison. Measurements of both strains have been taken in duplo.

DETAILED DESCRIPTION OF THE INVENTION

Surprisingly, we have now found that yeasts expressing a heterologous gene for TPS are showing a higher fermentative capacity, resulting in increased CO₂ and ethanol production rates by having an increased carbohydrate metabolic capacity. In our experiments no difference was seen in batch cultures and levels of trehalose remained unaltered. However, in chemostate cultures, oscillations in metabolism were observed as noted by changes in oxygen consumption. When the

yeasts were cultured in an anaerobic culture after preculturing in aerobic culture under limited sugar conditions, which is a good model for the industrial production of baker's yeast in fed-batch cultures, these strains showed a dramatic increase in fermentation capacity.

5 It is known that trehalose synthesis in yeast is dependent on a complex of three enzymes, trehalose phosphate phosphatase (TPP), trehalose phosphate synthase (TPS) and a third enzyme, which harbours homologous regions to both TPP and TPS, a so-called bipartite enzyme (TPS/P). Said third enzyme has also been thought to have regulatory
10 functions (Thevelein, J.M., Hohmann, S., TIBS 20, 3-10, 1995). These three enzymes interact with each other to produce trehalose-6-phosphate and, subsequently, trehalose from UDP-glucose and glucose-6-phosphate. This complex is suggested to play a role in sugar sensing and signalling. Disturbance of this complex, modification of its
15 activity, or altered regulation of the activity by introduction of TPS appears to result in an increase of the glycolysis and thus in an increased rate of CO₂ and ethanol production.

The present invention provides a transformed microorganism,
20 preferably a yeast, which is able to express a TPS gene. This TPS gene is preferably of heterologous origin. By heterologous DNA is meant DNA not originating from the same yeast genus. For example, heterologous DNA is used when *Saccharomyces* is transformed with DNA not originating from *Saccharomyces*. The heterologous DNA may be of any origin, for
25 instance, bacterial, fungal, plant, animal or human DNA. Preferably the TPS gene is derived from *Escherichia coli*. This enzyme has been described in EP 0 784 095. Also other genes coding for TPS are available at this moment as can be learnt from WO 97/42326.

Preferably the expression of TPS is under control of a
30 constitutive promoter. Thus, in the case of overexpressing the endogenous TPS gene a recombinant DNA construct should be introduced which is able to place the endogenous gene under control of a constitutive promoter. By a constitutive promoter is meant a promoter which effects expression of a gene independently of environmental
35 conditions, for example the alcohol dehydrogenase promoter (*ADH1*-promoter) similar to that described by Bennetzen, J.C. and Hall, B.D. (J. Biol. Chem. 257, 3018), the glyceraldehyde-3-phosphate dehydrogenase promoter (*GAPDH*-promoter), similar to that as described

by Holland and Holland (Holland and Holland, (1980), J. Biol. Chem. 255, 2596-2605). Use of such a promoter effects expression under the conditions of fed-batch fermentation production processes as well as under dough conditions.

5 The transformed yeast according to this invention can be used as a starting strain for strain improvement procedures, such as mutation, mass mating and protoplast fusion. The resulting strains are considered to form part of this invention.

10 After introduction of gene coding for TPS an increase in gas production may be observed in doughs.

15 The invention not only applies to dough, but to any fermentation process, for example fermentation systems for industrial ethanol production from hydrolysed starch. Transformed yeast strains of this invention therefor include not only strains of baker's yeast, but also, for example, beer, whiskey and wine yeast strains.

20 Next to TPS other enzymes known to be part of or influence the trehalose synthesis pathway can be used to mimic the effects of expression of TPS. It is thought that the effects of TPS on the carbohydrate metabolism of the yeast are produced by its effect on the amounts of intracellular trehalose-6-phosphate (T-6-P). TPS is capable of forming T-6-P from glucose-6-phosphate and UDP-glucose. It is envisaged that any enzymes which are able to form T-6-P or to prevent its degradation would have the same effects. Thus production of an anti-sense trehalose phosphate phosphatase (as-TPP) which would result in the inhibition of degradation of T-6-P and thus in increased T-6-P levels, would have a similar effect.

25 Trehalase is an enzyme which is capable of degrading trehalose. It has been shown in plants (WO 97/42326) that expression of a DNA construct that prevents the functioning of endogenous trehalase (as-trehalase) yields phenotypical changes which are also seen with expression of heterologous TPS. Thus, it is envisaged that anti-sense expression of trehalase in yeast would give similar results as the expression of TPS.

30 Budapest treaty deposits were made with the Centraalbureau voor Schimmelcultures (Baarn, the Netherlands) for yeast strain S. cerevisiae harbouring pMOG1199 under number CBS 923.97 and S.

cerevisiae harbouring pMOG1198 under number CBS 922.97 at Monday, 7 July 1997.

EXPERIMENTAL PART

DNA manipulations

For all DNA procedures (DNA isolation from *E.coli*, restriction, ligation, transformation, etc.) references is made to the handbook of Sambrook et al. (Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning; a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Strains

E. coli K-12 strain DH5-alpha is used for cloning. Yeast strain *Saccharomyces cerevisiae* CEN.PK113-3C MATa *trp1-289 MAL2-8^c SUC2* is used in all examples described.

Construction of pMOG1199

A DNA fragment harbouring the *TPS E. coli* coding sequence including the plant 3' poly adenylation signal was obtained by digesting pMOG799 (PCT/EP 97/02497) with the restriction enzymes *SmaI* and *PstI*. This fragment was inserted in the yeast shuttle vector p424 GPD (Mumberg, D., Müller, R. and Funk, M., Gene 156, 119-122, 1995), also digested with *SmaI* and *PstI*, yielding pMOG1199.

Construction of pMOG1198

Similar to the cloning of pMOG1199, a DNA fragment harbouring the *TPP E. coli* coding sequence was obtained tailored in such a way that *SmaI* and *PstI* sites are present at the terminal ends. This fragment was inserted in the yeast shuttle vector p424 GPD (Mumberg, D., Müller, R. and Funk, M., Gene 156 (1995) 119-122), also digested with *SmaI* and *PstI*, yielding pMOG1198.

Electroporation to Yeast

Both constructs pMOG1198 and pMOG1199 were transferred to yeast strain *Saccharomyces cerevisiae* CEN.PK113-3C MATa *trp1-289 MAL2-8^c SUC2* by electroporation (In: Evans, I.H., Methods in Molecular Biology: Yeast protocols pp.139-145, 1996, Humana Press, Totowa, New Jersey)

CULTURING METHODS

Strain maintenance

Precultures of strains were grown to stationary phase in shake-flask cultures on mineral medium containing 2% (w/v) glucose. After adding
5 glycerol (30% v/v), 2 ml aliquots were stored in sterile vials at -80°C. These frozen stock cultures were used to inoculate precultures for batch and chemostat cultivation.

Media

10 A defined mineral medium containing vitamins was prepared as described by Verduyn et al. (1992) was used. For chemostat cultivation, the glucose concentration in reservoir media was 7.5 g.l⁻¹ (0.25 mol C.l⁻¹).
1)

Shake-flask cultivation

15 Precultures were prepared by inoculating 100 ml mineral medium (0.3% w/v glucose) with 1 ml frozen stock culture. Cultures were incubated on an orbital shaker (200 rpm) at 30°C for 1 day. For growth curves, 4 ml of preculture was inoculated in a 500 ml Erlenmeyer flask with 100
20 ml mineral medium (2% w/v glucose or 1% v/v ethanol, pH 6.0) and then shaken (200 rpm) at 30°C. Optical-density measurements were performed at appropriate time intervals as described by Weusthuis, R.A. et al. (Is the Kluyver effect in yeast caused by product inhibition?
Microbiology 140: 1723-1729, 1994).

Chemostat cultivation in fermenters

Aerobic chemostat cultivation was performed at 30°C in laboratory fermenters (Applikon, Schiedam, The Netherlands), at a stirrer speed of 800 rpm and a dilution rate of 0.10 h⁻¹. The working volume of the cultures was kept at 1.0 l by a peristaltic effluent pump coupled to an electrical level sensor. This set-up ensured that under all growth conditions, biomass concentrations in samples taken directly from the cultures differed by less than 1% from biomass concentrations in samples taken from the effluent line. The pH was kept at 5.0 ± 0.1 by an ADI 1030 biocontroller, via the automatic addition of 2 mol/l⁻¹ KOH. The fermenter was flushed with air at a flow rate of 0.5 l/min⁻¹ using a Brooks 5876 mass-flow controller. The dissolved oxygen concentration was continuously monitored with an oxygen electrode (Ingold, 34 100 3002) and remained above 60% of air saturation. Chemostat cultures were routinely checked for purity using phase-contrast microscopy.

ANALYSESGas analysis

The exhaust gas was cooled in a condenser (2°C) and dried with a Perma Pure dryer (PD-625-12P). O₂ and CO₂ concentrations were determined with a Servomex 1100A analyzer and a Beckman model 864 infrared detector, respectively. The exhaust gas flow rate was measured as described by Weusthuis et al., (1994). Specific rates of CO₂ production and O₂ consumption were calculated according to the method of van Urk, H. et al. (Metabolic responses of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 upon transition from glucose limitation to glucose excess. Yeast 4: 283-291, 1988).

Determination of culture dry weight

Culture samples (10 ml) were filtered over preweighed nitrocellulose filters (pore size 0.45 µm; Gelman Sciences). After removal of medium, the filters were washed with demineralized water, dried in a Sharp R-4700 microwave oven for 20 min at 360 W output, and weighed. Parallel samples varied by less than 1%.

Determination of fermentative capacity

Samples containing exactly 100 mg dry weight of biomass from a steady-state chemostat were harvested by centrifugation at 5000g for 5 min, washed once and resuspended in 5 ml 0.9% (w/v) NaCl solution.

Subsequently, these cell suspensions were introduced into a
5 thermostated (30°C) vessel containing 10 ml 5-fold concentrated mineral medium, set at pH 5.6. The volume was adjusted to 40 ml with demineralized water. After 10 min incubation a 10 ml glucose pulse (100 g.l⁻¹) was given and samples (1 ml) were taken at appropriate
10 time intervals. The working volume was 50 ml with a 10 ml headspace which was continuously flushed with CO₂ gas at a flow rate of approximately 10 ml.h⁻¹. The ethanol concentration in the supernatant was determined with a colorimetric assay according to Verduyn, C. et al. (Colorimetric alcohol assays with alcohol oxidase. J. Microbiol. Meth. 2: 15-25, 1984) using partially purified alcohol oxidase from
15 Hansenula polymorpha. The fermentative capacity is expressed as mmol ethanol produced (g dry weight)⁻¹.h⁻¹.

Metabolite analysis

Glucose in reservoir media and supernatants was determined
20 enzymatically using the GOD-PAP method (Merck systems kit 14144. The ethanol concentration in the medium was determined with a colorimetric assay according to Verduyn, C. et al. (1984) using partially purified alcohol oxidase from Hansenula polymorpha.

25

EXAMPLE 1

Growth-velocities and yield of the different transgenic *Saccharomyces* strains were determined in shake-flask experiments using mineral medium and glucose or ethanol as carbon source. Strains used are a wild-type *Saccharomyces* strain (prototrophic growth, WT), an identical strain harbouring an empty expression vector (EV), harbouring pMOG1199 (TPS) or pMOG1198 (TPP). Velocities of growth are depicted in **Table 1**.

Table 1

Velocity of growth (OD. hour ⁻¹)	WT	EV	TPS	TPP
Glucose (n=2)	0.42±0.0 3	0.37±0.0 3	0.34±0.0 2	0.34±0.02
Ethanol (n=2)	0.16±0.0 2	0.15±0.0 2	0.15±0.0 1	0.16±0.01

The final optical density of the batch-grown cultures has been measured as an indication for biomass yield. Results are depicted in **Table 2**.

Table 2

Final OD	WT	EV	TPS	TPP
Glucose (n=2)	6.3 ± 0.2	6.0 ± 0.2	5.0 ± 0.4	5.3 ± 0.1

As can be concluded from these data, the transgenic strains harbouring TPS and TPP grow only slightly less fast on glucose compared to the wild-type and empty vector strain resulting a slightly reduced final biomass in batch-grown cultures. These data suggest there is no strong effect on processes like aerobic fermentation. Using ethanol as a carbon source, no significant difference was found between the strains tested.

EXAMPLE 2

The different yeast strains were also compared on growth characteristics in continuous fed aerobic chemostat cultures. When grown in steady state, no differences were noted between the different strains in biomass yield or production of metabolites. Remarkably, the TPS transgenic *Saccharomyces* strains revealed a persistent and characteristic metabolic oscillation as noted by continuous measuring the soluble oxygen concentration in the cultures. This oscillation was not comparable to that observed when spontaneous synchronisation of the cell-cycle occurs in some wild-type *Saccharomyces* strains, indicating that some form of metabolic regulation is disturbed in the transgenic strains.

EXAMPLE 3

The TPS transgenic yeast strain was also tested in a system that reflects the conditions occurring during the preparation of dough. For this purpose, the strain was precultured in aerobic, sugar limited chemostat cultures. This preculture reflects the industrial production process of bakers-yeast in fed-batch cultures. Subsequently the culture was transferred to anaerobic conditions in the presence of sugar to monitor the production of ethanol and CO₂. The results of those experiments are depicted in **figure 1**. Strikingly, in repeated separate experiments, an increase of 30-40% of the fermentation capacity was noted in the transgenic TPS strain compared to the wild-type and control strains.

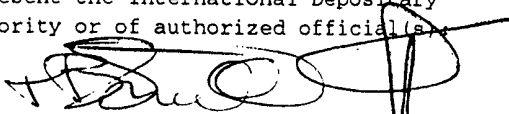
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RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Mogen International N.V.
Einsteinweg 97
2333 CB LEIDEN
Nederland

name and address of depositor

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
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identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: S. cerevisiae harbouring pMOG 1198	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CBS 922.97
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☒ a scientific description ☐ a proposed taxonomic designation <i>(mark with a cross where applicable)</i>	
III. RECEIPT AND ACCEPTANCE	
This International Depositary accepts the microorganism identified under I above, which was received by it on Monday, 7 July 1997 <i>(date of the original deposit)¹</i>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on not applicable <i>(date of the original deposit)</i> and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on not applicable <i>(date of receipt of request for conversion)</i>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Centraalbureau voor Schimmelcultures Address: Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s)  Mrs F.B. Snippe-Claus dr R.A. Samson Date: Monday, 14 July 1997

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

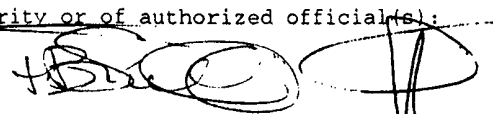
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INTERNATIONAL DEPOSITARY AUTHORITY
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I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: S. cerevisiae harbouring pMOG 1199	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CBS 923.97
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☒ a scientific description ☐ a proposed taxonomic designation <i>(mark with a cross where applicable)</i>	
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V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Centraalbureau voor Schimmelcultures Address: Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Mrs F.B. Snippe-Claus dr R.A. Samson Date: Monday, 14 July 1997

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

CLAIMS

1. Microorganism having modified carbohydrate metabolism and/or fermentation capacity characterized in that it comprises a recombinant DNA capable of expressing trehalose phosphate synthase (TPS).

2. Microorganism according to claim 1, characterized in that the recombinant DNA is heterologous, preferably selected from the group of bacterial, fungal, plant, animal and human DNA, more preferably from *Escherichia coli*.

3. Microorganism according to claim 1 or 2, characterized in that it is a yeast, preferably a yeast of a strain of *Saccharomyces*, more preferably *Saccharomyces cerevisiae*.

4. Microorganism having a modified carbohydrate metabolism and/or fermentation capacity characterized in that this alteration is caused by a recombinant DNA expressing a product which influences the endogenous level of trehalose-6-phosphate, the product preferably selected from the group consisting of TPP, TPS, trehalase, and anti-sense trehalase.

5. Method for producing a microorganism having an increased carbohydrate metabolism and/or fermentation capacity characterized in that the microorganism is transformed with a recombinant DNA capable of expression of an expression product which influences the endogenous level of trehalose-6-phosphate.

6. Method according to claim 5, characterized in that the product is selected from the group consisting of TPP, TPS, trehalase, and anti-sense trehalase.

7. Use of a microorganism according to any of claims 1-4 in a fermentation process.

8. Dough comprising a microorganism according to any of claims 1-4.

9. Method for baking, characterized in that a dough according to claim 8 is used.

10. Bread or other bakery products, characterized in that they are produced from the dough according to claim 8 and/or through the method according to claim 9.

11. Method for the production of ethanol, characterized in that a microorganism according to any of claims 1-4 is used.

12. Method for producing alcoholic beverages, characterized in that a microorganism according to any of claims 1-4 is used in the fermentation.

13. Beer or other alcoholic beverages, characterized in that they are produced according to the method of claim 12.

14. Microorganism as deposited under number CBS 923.97 at the Centraal Bureau of Schimmelcultures on July 7, 1997.

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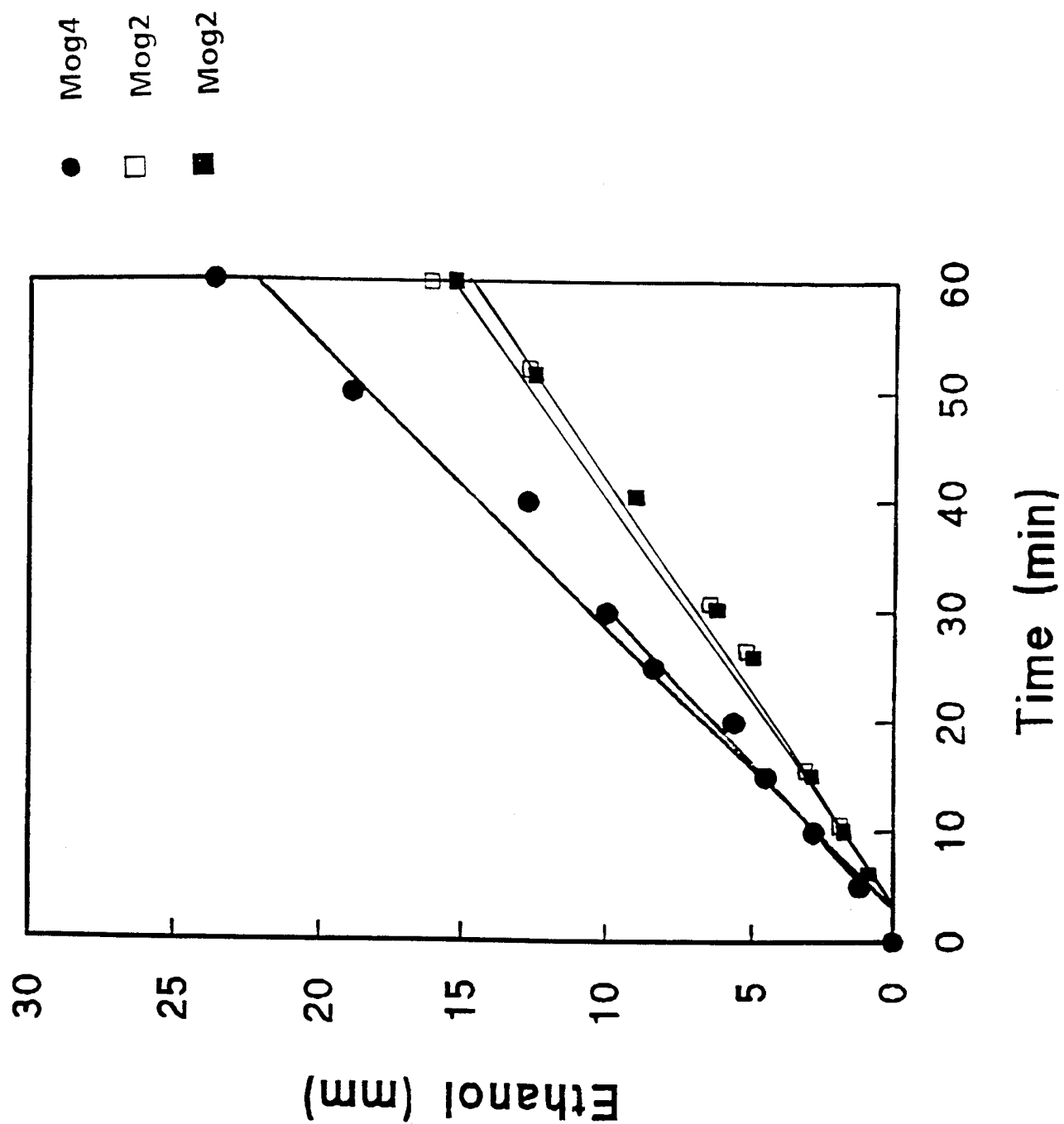


Fig. 1

BUDAPEST TREATY ON THE INTERNATIONAL
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 FOR THE PURPOSES OF PATENT PROCEDURE **WIPO**

INTERNATIONAL FORM

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*name and address of the party to whom the
 viability statement is issued*

VIABILITY STATEMENT
 issued pursuant to Rule 10.2 by the
 INTERNATIONAL DEPOSITARY AUTHORITY
 identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Mogen International N.V. Address: Einsteinweg 97 2333 CB LEIDEN Nederland	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CBS 922.97 Date of the deposit or of the transfer: ¹ Monday, 7 July 1997
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on Friday, 11 July 1997 ² . On that date, the said microorganism was ☒ ³ viable ☐ ³ no longer viable	

¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY HAS BEEN PERFORMED⁴

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Centraalbureau voor Schimmelcultures

Address: Oosterstraat 1
P.O. Box 273
3740 AG BAARN
The NetherlandsSignature(s) of person(s) having the power to
represent the International Depositary
Authority or of authorized official(s):

Mrs F.B. Snippe-Claus

dr R.A. Samson

Date: Monday, 14 July 1997

⁴ Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Mogen International N.V.
Einsteinweg 97
2333 CB LEIDEN
Nederland

*name and address of the party to whom the
viability statement is issued*

VIABILITY STATEMENT

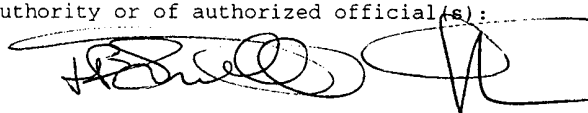
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Mogen International N.V. Address: Einsteinweg 97 2333 CB LEIDEN Nederland	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CBS 923.97 Date of the deposit or of the transfer:¹ Monday, 7 July 1997
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on Friday, 11 July 1997². On that date, the said microorganism was ☒³ viable ☐³ no longer viable	

¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Centraalbureau voor Schimmelcultures Address: Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Mrs F.B. Snippe-Claus dr R.A. Samson Date: Monday, 14 July 1997

⁴ Fill in if the information has been requested and if the results of the test were negative.