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(57) Abstract: Described herein are new approaches for the selection of *S. cerevisiae* strains with increased ethanol production from hydrolyzed starch derived sugars. An industrial production strain of *Saccharomyces cerevisiae* AS400 was subjected to positive selection of mutants resistant to toxic concentrations of oxythiamine, trehalose, 3-bromopyruvate, glyoxylic acid, and glucosamine. The selected mutants are characterized by 5-8% increase in ethanol yield (g g⁻¹ of consumed glucose) as compared to the parental industrial ethanol-producing strain. A multiple-step selection approach that consisted of the sequential selection using glyoxylic acid, glucosamine and bromopyruvate as selective agents resulted in a 12% increase in ethanol yield during fermentation on industrial media. These results indicate that the selection methods provided herein are useful for producing a variety of strains that are promising candidates for industrial ethanol production.



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METHODS FOR THE POSITIVE SELECTION OF ETHANOL OVERPRODUCING MUTANTS FROM SACCHAROMYCES CEREVISIAE

5 CROSS REFERENCE TO RELATED APPLICATION[S]

[0001] This Application claims priority to US provisional application No. 62/145,606 entitled “NEW METHODS FOR THE POSITIVE SELECTION OF ETHANOL OVERPRODUCING MUTANTS FROM SACCHAROMYCES CEREVISIAE” filed April 10, 2015 which is incorporated herein by reference in its
10 entirety.

BACKGROUND OF THE INVENTION

[0002] Today the biofuel industry primarily produces ethanol from traditional feedstocks such as cereal crops like corn and wheat and sugar crops like sugar cane and
15 sweet sugar beet.

[0003] The fermentation of plant carbohydrates by yeast dates back to the Neolithic period [1]. Today, yeasts are widely used in many biotechnological processes, with the largest use for the production of alcoholic beverages such as beer and wine and fuel ethanol. The yeast *Saccharomyces cerevisiae* is the predominant microorganism for
20 industrial ethanol production. This yeast is characterized by several desirable industrial properties which include fast growth, efficient glucose anaerobic metabolism, high ethanol productivity and yield, and tolerance to several environmental stress-factors, such as high ethanol concentration and low oxygen level [2]. In spite of good ethanol yields from glucose and sucrose, the yield from these sugars can be further increased. There are
25 several approaches that can be employed for this, which include genetic engineering techniques that target the redirection of yeast metabolism towards increased yield of ethanol production from carbohydrates by reducing co-product generation [3]. Particularly, this can be achieved through the reduction of glycerol and biomass synthesis, since these represent the major by-products made during a bioethanol production. During
30 anaerobic growth of *S. cerevisiae*, glycerol serves as an essential electron sink for re-oxidizing reduced NADH generated during biosynthesis of ethanol. The employment of engineering approaches, which aim to change cellular redox metabolism via reduced

formation of cytosolic NADH, resulted in the reduction of glycerol production with increase in ethanol yield [4, 5, 6, 7]. Decreasing biomass accumulation is an alternative way to increase ethanol yield from sugar consumed. The extent of biomass accumulation is dependent on the availability of energy in the form of ATP. If cellular ATP yield is decreased, it was anticipated that an increase in ethanol yield will result [8]. As predicted, increase in ethanol production was demonstrated using metabolic engineering approaches via the introduction of ATP-hydrolyzing futile cycles in yeast through the deregulation of some of the gluconeogenic enzymes [9]. A positive effect on ethanol production was also accomplished by the overexpression of ATPase [10] or the alkaline phosphatase Pho8 which can also hydrolyze ATP [11].

[0004] Though redirecting yeast metabolism towards increasing ethanol production using genetic engineering approaches is relatively easy to achieve, the usage of genetically modified strains for ethanol production, mainly in wine yeast, is not accepted in many parts of the world due to consumer's resistance to the use of genetically modified organisms (GMO) for beverage alcohol [12]. For this reason, the use of non-GMO approaches, which consist of traditional selection and adaptive evolution, must be relied on developing new improved strains [13]. Traditional selection and adaptive evolution involve applying culture conditions that provide a selection pressure favoring the growth of mutants within a population that confer the trait of interest. Thus, the culturing of cell populations in a specific selective environment will direct the accumulation of adaptation towards a desired phenotype [13, 14, 15]. There are several excellent examples that illustrate the usefulness of this approach to the selection of anaerobical xylose utilizing yeast strains [16], yeast with enhanced maltose utilization and osmotolerance [17], yeast with enhanced ethanol tolerance [18] and yeast with improved fermentation rate with decreased formation of acetate [19].

[0005] In the present disclosure, there is described new methods for positive selection of *S. cerevisiae* strains with enhanced ethanol production phenotypes. The methods are exemplified using industrial *S. cerevisiae* AS400 strain that is used for bioethanol production at Archer Daniels Midland Company (Decatur, IL, USA). The selective agents oxythiamine, trehalose, 3-bromopyruvate, glyoxylic acid and glucosamine, were used in this description and chosen for their inhibitory effect on the enzymes involved in alcoholic fermentation and stress response.

SUMMARY OF THE INVENTION

[0006] The present disclosure describes a method of making a *S. cerevisiae* strain with enhanced ethanol producing characteristics comprising, contacting a culture of a parent strain of *S. cerevisiae* with a sufficient amount of a first selection agent selected from the group consisting of oxythiamine, trehalose, bromopyruvate, glyoxylic acid and glucosamine for the selection agent to be toxic to the parent strain; growing the contacted culture on a plating medium containing a sugar as a carbon source; selecting candidadate spontaneous mutant strains of the culture that grow on the plating medium, and measuring an ethanol producing characteristic of the candidate spontaneous mutant strains to determine whether the candidate strain demonstrates enhanced ethanol production characteristics in comparison to the parent strain and if so, obtaining the *S. cerevisiae* strain with enhanced ethanol producing characteristics.

[0007] Certain embodiments include a method wherein the obtained spontaneous mutant strain with enhanced ethanol production characteristics is contacted with an amount of a second selection agent selected from the group consisting of oxythiamine, trehalose, bromopyruvate, glyoxylic acid and glucosamine for the selection agent to be toxic to the strain, wherein the second selection agent differs from the first selection agent; and repeating the growing, selecting and measuring acts to obtain a second spontaneous mutant strain having enhanced ethanol production characteristics in comparison to the first obtained spontanerous mutant strain.

[0008] Further embodiments include a method wherein the enhanced ethanol producing characteristic is at least one of: increased ethanol production measured as wt ethanol produced per volume, increased ethanol productivity measured as wt ethanol produced per volume per unit time, increased ethanol yield defined by wt ethanol produced per wt sugar fed to the strain, and increased specific ethanol productivity defined by wt ethanol produced per weight *S. cerevisiae* biomass per unit time.

[0009] Another aspect of the present disclosure includes a *S. cerevisiae* strain with enhanced ethanol producing characteristics made by any of the methods as

described this far. Certain embodiments include a *S. cerevisiae* strain that is an industrial ethanol production strain. Specific exemplary embodiments include a *S. cerevisiae* strain selected from the group consisting of AS400-567, AS400-543, AS400-617, AS400-510, AS400-128, AS400-510-42, and AS400-42-214. Other embodiments
5 include a *S. cerevisiae* strain derived from a parent selected from the group consisting of AS400-567, AS400-543, AS400-617, AS400-510, AS400-128, AS400-510-42, and AS400-42-214.

[0010] Additional aspects of the invention include a method of producing ethanol comprising fermenting a sugar with a *S. cerevisiae* strain selected from the
10 group consisting of AS400-567, AS400-543, AS400-617, AS400-510, AS400-128, AS400-510-42, and AS400-42-214 or a strain derived from such. Further embodiments include a method of separating the ethanol produced by the fermentation of a strain selected from the group consisting of AS400-567, AS400-543, AS400-617, AS400-510, AS400-128, AS400-510-42, and AS400-42-214 or a strain derived from such.

15 [0011] Another aspects of the present invention includes a method of making a *S. cerevisiae* strain with enhanced ethanol producing characteristics comprising, contacting a culture of a parent strain of *S. cerevisiae* with a sufficient amount of a first selection agent selected from the group consisting of oxythiamine, trehalose, glycoxylic acid and glucosamine for the selection agent to be toxic to the parent strain; growing the
20 contacted culture on a plating medium containing a sugar as a carbon source; selecting candidate spontaneous mutant strains of the culture that grow on the plating medium, and measuring an ethanol producing characteristic of the candidate spontaneous mutant strains to determine whether the candidate strain demonstrates enhanced ethanol production characteristics in comparison to the parent strain and if so, obtaining the *S.*
25 *cerevisiae* strain with enhanced ethanol producing characteristics.

[0012] An additional aspect of the invention includes a method wherein the obtained spontaneous mutant strain with enhanced ethanol production characteristics that has been contacted with a first selection agent selected from the group consisting of
30 oxythiamine, trehalose, glycoxylic acid and glucosamine is further contacted with an amount of a second selection agent selected from the group consisting of oxythiamine,

trehalose, glyoxylic acid and glucosamine for the selection agent to be toxic to the strain, wherein the second selection agent differs from the first selection agent; and repeating the growing, selecting and measuring acts to obtain a second spontaneous mutant strain having enhanced ethanol production characteristics in comparison to the first obtained spontaneous mutant strain.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] All of the figures depict growth, glucose consumption and ethanol production during alcoholic fermentation of mutants and initial AS400 strains. Several strains were cultured under semi anaerobic conditions in YNB medium with 200 g l⁻¹ D-glucose and were incubated at 34°C, shaking at 120 rpm using. The squares, triangles and circles of each figure correspond to biomass accumulation (g of biomass l⁻¹ of medium), glucose consumption (g of glucose l⁻¹ of medium) and ethanol production (g of ethanol l⁻¹ of medium), respectively. Additional information can be found in Table 1 about each of these fermentations.

[0014] Figure 1 depicts a fermentation as described above using the strain AS400.

[0015] Figure 2 depicts a fermentation as described above using the strain AS400-567.

[0016] Figure 3 depicts a fermentation as described above using the strain AS400-543.

[0017] Figure 4 depicts a fermentation as described above using the strain AS400-617.

[0018] Figure 5 depicts a fermentation as described above using the strain AS400-510.

[0019] Figure 6 depicts a fermentation as described above using the strain AS400-128.

[0020] Figure 7 depicts a fermentation as described above using the strain AS400-510-42.

[0021] Figure 8 depicts a fermentation as described above using the strain AS400-510-42-214.

DETAILED DESCRIPTION OF THE INVENTION

[0022] The *S. cerevisiae* strain AS400 (Archer Daniels Midland Company (Decatur, IL, USA)) was used as the parental strain for the selection of improved strains.

5 All chemicals used through the work were obtained from Sigma, USA, unless otherwise noted.

[0023] *S. cerevisiae* strains were routinely cultured at 30°C. Yeast strains were maintained in rich YPD (1% yeast extract, 1% peptone and 2% glucose) or mineral YNB (0.67%, yeast nitrogen base without amino acids, (Difco, USA), 0.5% ammonium sulfate, 2% glucose) media. *S. cerevisiae* cells were grown overnight in YPD and washed twice with H₂O. For selection of ethanol overproducing mutants, a cell suspension of AS400 (10⁶, 1.7 x 10⁶ and 3.3 x 10⁶ cells per plate $d = 90$ mm) was plated on YNB solid medium with glucose (2%) as the carbon source supplemented with toxic concentrations of the selective agents, oxythiamine (118 mM), trehalose (1.31 M), 3-bromopyruvate (2.75 mM), glyoxylic acid (4 mM) or glucosamine (2.75 mM) and incubated for 7-14 days depending on selective agents. The minimal toxic concentrations of inhibitors were defined as the amount of the agent in YNB-glucose solid medium that totally inhibited growth of AS400 strain after plating 3.3 x 10⁶ cells per plate. Single yeast colonies resistant to the selective agents were picked up, re-streaked on fresh YNB plates containing corresponding concentrations of reagent and used in fermentation experiments. The selected mutants were stable, maintaining resistance to the selective agents. Selected strains were also found to be stable in respect of their ability to produce increased amount of ethanol after 6 months of storage at -80 °C. No cross-resistance to the different selection agents used was found in the mutants.

25 [0024] Corn steep liquor (CSL) is a by-product of corn wet-milling that is obtained after initial soaking corn grain in water prior to milling. CSL has been used as an effective low-cost fermentation nutrient supplement in several different fermentations [20]. CSL was used to supplement hydrolyzed corn meal as routinely used in industrial alcoholic fermentation processes in distillery and fuel-ethanol plants.

30 [0025] The CSL medium was prepared by combining two solutions that were prepared as described. Solution 1 was prepared by adding 200 g of corn meal (Melvit, Poland) mixed with 800 ml of deionized water and pH adjusted to 6.0 using NaOH. The

enzyme alpha-amylase (Liquizyme SC) was added at a rate of 0.1 units of per gram of meal, and the slurry was liquified by heating the mixture to 80°C for 30 minutes. To prepare Solution 2, 63 ml of the CSL concentrate (provided by ADM Company) containing ~50% dry solids was mixed with 137 ml of deionized water. The composition of CSL was described elsewhere [21]. Prior to use, the two solutions were autoclaved, cooled, and then combined and mixed. The enzyme glucoamylase (Liquizyme SC) was added in the amount of 0.2 units of glucoamylase per gram of meal to sterile flasks, and the flasks were incubated at 28°C. Glucose concentration was measured in the control flasks without yeast inoculation at 200 g l⁻¹ in the CSL medium prepared.

[0026] For ethanol fermentation, all strains were tested using YNB medium that was supplemented with 20% glucose or by using a medium containing corn CSL supplemented with hydrolyzed corn meal as the primary carbon source.

[0027] For alcoholic fermentation, cells of AS400 strain and its derivatives were grown overnight in 100 ml of YPD medium in 300 ml Erlenmeyer flasks and then used to inoculate 20 ml aliquots of mineral YNB medium supplemented with 200 g l⁻¹ glucose or CSL medium supplemented with hydrolyzed meal in 50 ml Erlenmeyer flasks. An initial biomass concentration of 8 g (dry weight) l⁻¹ was used for fermentation in YNB medium. For CSL medium, an initial biomass concentration of 10 g (dry weight) l⁻¹ was used (this is the cell density normally used in industrial ethanol fermentation) [22]. Fermentation was carried out at a temperature of 34°C with limited aeration using a gyratory shaker at a setting of 120 rev. min⁻¹. Samples were taken every 3 hours for YNB medium or 12 hours for CSL medium. Fermentation experiments were performed in at least triplicate to ensure reproducibility.

[0028] The concentration of ethanol in fermentation media was determined using alcohol oxidase/peroxidase-based enzymatic kit “Alcotest” [23, 24]. The biomass was determined using turbidity with a Helios Gamma spectrophotometer (OD, 600 nm; cuvette, 10 mm) with gravimetric calibration. Glucose concentration was determined using the “Diagluco” assay kit (UBT, Lviv) [23]. All samples were assayed in duplicate.

[0029] Oxythiamine is a thiamine antagonist that, after conversion to oxythiamine pyrophosphate, binds to the active centers of thiamine-dependent enzymes. It was shown that *S. cerevisiae* cells treated by oxythiamine possessed higher specific activity of

transketolase and pyruvate decarboxylase after 12 h of cultivation [25]. Transketolase is a crucial enzyme of the pentose phosphate pathway, providing metabolic intermediate (glyceraldehyde-3-phosphate and fructose-6-phosphate) for glycolysis and the substrates (NADPH, ribose-phosphate) for macromolecule synthesis. Pyruvate decarboxylase in turn is the key enzyme directing carbon flux from pyruvate to ethanol. The present inventors speculated that yeast strains resistant to oxythiamine would possess increased pyruvate to acetaldehyde conversion that would result in increased production of ethanol. Several strains, derivatives of AS400, were therefore selected on the selective media supplemented with toxic concentration of oxythiamine (118 mM). Among these strains, AS400-567 was demonstrated to in fact have an increase in ethanol synthesis during fermentation on YNB medium. This strain accumulated 6% more ethanol relative to the parental strain AS400 during fermentation producing 85.2 g l⁻¹ of ethanol versus 80.3 g l⁻¹ for AS400 (Table 1, Fig. 1. And Fig. 2). Due to similar biomass accumulation and complete glucose consumption during fermentation, ethanol productivity (4.73 g l⁻¹ h⁻¹), specific ethanol productivity (1.97 g g⁻¹ biomass h⁻¹) and ethanol yield (0.426 g of ethanol g⁻¹ of consumed glucose) for strain AS400-567 were also increased for 6% as compared to AS400. Alcoholic fermentation in CSL medium demonstrated a 7% increase in ethanol production for strain AS400-567 for a total ethanol production of 90.5 g l⁻¹, as compared to strain AS400 which produced 84.6 g l⁻¹ of ethanol (Table 1). Ethanol yield for strain AS400-567 was also increased on 7% since this and parental strains consumed glucose completely (Table 1). Measurement of the productivity of ethanol synthesis per biomass for this and other strains was not carried out in CSL medium as it contains many insoluble particles that interfered with cell biomass measurement using direct dry weight or by optical density analyses.

[0030] During alcoholic fermentation, yeast cells are subjected to stress that could affect their viability and fermentation efficiency. Trehalose functions as one of the major stress protectants and the synthesis of trehalose is induced by several documented stress conditions at the transcriptional level in *S. cerevisiae* [26]. Trehalose-6-phosphate synthase is the key enzyme involved in trehalose synthesis and is known to be inhibited by high concentrations of trehalose [27]. Yeast mutants resistant to increased concentrations of trehalose may have elevated intracellular concentration of this disaccharide with subsequent beneficial effect of trehalose on ethanol production. Strain

AS400 was subjected to a selection on the medium containing toxic concentration of trehalose (1.31 M). Several trehalose resistant strains were selected after 14 days of incubation. The trehalose resistant strain AS400-543 had a 5% increase in ethanol production (84.3 g l⁻¹), ethanol productivity (4.68 g l⁻¹ h⁻¹) and an ethanol yield (0.422 g g⁻¹ of consumed glucose) as compared to the parental strain during fermentation on YNB medium (Table 1, Fig. 3). However, specific ethanol productivity of this strain was increased by 48%, reaching 2.75 g g⁻¹ biomass h⁻¹ as calculated from a 1.6 fold decrease in specific growth rate (Table 1, Fig. 3). A similar increase in ethanol production and ethanol yield was observed for this trehalose resistant strain during fermentation on industrial CSL medium. Strain AS400-543 synthesized a total of 88.8 g l⁻¹ of ethanol, whereas strain AS400 produced a total of 84.6 g l⁻¹ of ethanol (Table 1). Ethanol yield reached 0.444 g g⁻¹ of consumed glucose providing 5% increase as compared to the parental strain (Table 1).

[0031] The present inventors speculated that yeast mutants resistant to bromopyruvate may have an increase in glycolysis with elevated amount of synthesized ethanol. The strain AS400-617 was selected on a medium supplemented with toxic concentration of this selective agent (2.75 mM). The bromopyruvate resistant strain was characterized by having up to 5% increase of ethanol production (84.1 g l⁻¹), a productivity (1.95 g g⁻¹ biomass h⁻¹) and an ethanol yield (0.421 g of ethanol g⁻¹ of consumed glucose) during fermentation on YNB medium when compared to the strain AS400 (Table 1, Fig. 4). Strain AS400-617 synthesized a total of 89.7 g l⁻¹ ethanol with up to 6% increase in ethanol accumulation when compared to initial strain AS400 in CSL medium (Table 1). Ethanol yield was also increased by 6% reaching 0.421 g g⁻¹ of the consumed glucose (Table 1).

[0032] Glyoxylic acid is a known irreversible inhibitor of pyruvate decarboxylase activity [31], the key enzyme in ethanol synthesis. Therefore, selection of ethanol overproducers was carried out using a YNB medium containing 4 mM glyoxylic acid. Several resistant colonies were obtained from AS400 after 5-8 days of plate incubation and subjected to testing for ethanol production from glucose. The isolated strain AS400-510 had a 6% elevation of total ethanol production (85.1 g l⁻¹), a productivity (1.97 g g⁻¹ biomass h⁻¹) and an increase in ethanol yield (0.426 g g⁻¹ of consumed glucose) during alcoholic fermentation in YNB medium (Table 1, Fig. 5). During fermentation on CSL

medium, a total of 91.4 g l⁻¹ of ethanol was produced with an ethanol yield (0.457 g g⁻¹ of consumed glucose) for an 8% increase in ethanol production and yield over AS400 (Table 1).

[0033] Glucosamine (2-deoxy-2-aminoglucose) is a structural analog of glucose.

5 Due to the absence of hydroxyl at the second position, this compound cannot be isomerized to the ketohexose (analog of fructose) by the glycolytic enzyme phosphoglucosomerase [32]. This leads to disruption of the glycolysis at this step. The glucosamine resistant mutants can be defective in glucose uptake, but this is very unlikely during the selection on glucose as the sole carbon source. Other explanation for this could
10 be the derepression of PGI1 only or the entire regulon involved in regulation of the majority of glycolytic enzymes. The present inventors speculated that this last event would result in an increase of glucose flux through glycolysis pathway. Glucosamine was therefore used as selective agents for positive selection of *S. cerevisiae* ethanol overproducers. Toxic concentrations of this selective agent was found to be 2 mM.
15 Strains AS400 and its derivative AS400-510 isolated as a glyoxylic acid resistant mutant were subjected for selection on a medium containing glucosamine. The selection on the YNB medium containing 2 mM of glucosamine resulted in the isolation of several resistant mutants after 14 days of incubation. The selected mutants were tested for ethanol production from glucose. The glucosamine resistant mutant derived from the AS400
20 (strain AS400-128) had an 5% increase in ethanol production (84.6 g l⁻¹), higher ethanol productivity (4.7 g l⁻¹ h⁻¹) and an increase in ethanol yield (0.423 g g⁻¹ of consumed glucose), while the specific ethanol productivity (2.24 g g⁻¹ biomass h⁻¹) was increased by 20% due to reduction of its specific growth rate (0.117 g l⁻¹ h⁻¹) by 12%, as compared to the parental strain, during fermentation on YNB medium (Table 1, Fig. 6). The highest
25 ethanol titer for strain AS400-128 during CSL fermentation was 89.6 g l⁻¹, whereas the initial strain synthesized 84.6 g l⁻¹ of ethanol, providing 6% increase of ethanol production and ethanol yield (Table 1). The strain AS400-510-42, that was derived from AS400-510, had an 8% increase in ethanol production (86.7 g l⁻¹), a specific ethanol productivity (2.01 g g⁻¹ biomass h⁻¹) and ethanol yield (0.434 g g⁻¹ of consumed glucose) as compared
30 to the AS400 during fermentation on YNB medium (Table 1, Fig. 7). Strain AS400-510-42 synthesized 93.0 g l⁻¹ of ethanol, while the initial strain AS400 produced only 84.6 g

l⁻¹ of ethanol during fermentation on CSL medium (Table 1). Thus, the increase of ethanol production and ethanol yield for this strain reached 10%.

[0034] It was further speculated that mutants obtained by sequential selection for resistance to two of the present selection agents may further increases in ethanol production characteristics. A double cross selection for glyoxylic acid and glucosamine resistance showed an increase of the ethanol production. This strain was labeled AS400-510-42. These results encouraged inventors to perform an additional round of the selection. Strain AS400-510-42 was used for the selection of bromopyruvate resistant derivatives. Several mutants were selected on the medium supplemented with 2.75 mM of bromopyruvate. Among them, strain AS400-510-42-214 which had a 10% increase in ethanol production (88.3 g l⁻¹), an ethanol productivity (4.91 g l⁻¹ h⁻¹) and an ethanol yield (0.442 g g⁻¹ of consumed glucose), while the specific ethanol productivity (2.34 g g⁻¹ biomass h⁻¹) was increased by 26% as a result of reduction of the specific growth rate (0.117 g l⁻¹ h⁻¹) on 12%, during fermentation on YNB medium (Table 1, Fig. 8). Strain AS400-510-42-214 had the highest amount of synthesized ethanol among all isolated mutants reaching a total of 94.8 g l⁻¹ of ethanol production on CSL medium. Strain AS400-510-42-214 showed a 12% increase in ethanol production and ethanol yield when compared to the parental strain AS400 (Table 1).

[0035] Applying the new simple positive methods for selection of *S. cerevisiae* strains with increased ethanol production on industrial medium supplemented with hydrolyzed corn meal, the inventors were able to demonstrate an increase in ethanol synthesis from 5 to 8% when compared to the parental industrial strain. The 5% increase in ethanol synthesis and ethanol yield was reached for strain selected on toxic concentration of trehalose, while mutants selected on bromopyruvate or glucosamine, oxythiamine and glyoxylic acid possessed 6%, 7% and 8% increase in ethanol production and yield, respectively. The combination of glyoxylic acid and glucosamine or glyoxylic acid, glucosamine and bromopyruvate for two- or three-steps selection schemes led to 10 or 12% increase of ethanol production and yield as compared to the parental strain. Strains selected on trehalose, glucosamine or glyoxylic acid, glucosamine and bromopyruvate for one- or three-step selections showed an increase in specific ethanol productivity, which is a result of the decrease in biomass accumulation during fermentation procedure. Selected strains described in this work possessed very similar

kinetics for glucose consumption. While not being bond by theory, it is possible that the observed increase in ethanol accumulation is caused by a decrease in accumulation of fermentation by-products, e.g. biomass, glycerol or acetate. The identification of the mutated genes in the selected mutants is the subject of our subsequent work.

- 5 **[0036]** For each type of mutants, more than 30 selected strains were selected and evaluated for the ethanol production from glucose. From 55 to 65% of isolated mutants resistant to the applied selective agents showed an increase in ethanol synthesis. On average, the ethanol yield in the analyzed strains was increased by 5-6% as compared to the parental strain. Relatively high percentage of ethanol overproducers lead us to
- 10 conclude that our approaches represent new methods for positive selection of ethanol overproducers.

[0037] Table 1: Growth rate, ethanol production, productivity, specific productivity and yield of *S. cerevisiae* mutants resistant to oxythiamine, trehalose, bromopyruvate, glyoxylic acid, glucosamine and initial strain AS400 during alcoholic fermentation of on YNB medium supplemented with 20% glucose and CSL medium with hydrolyzed meal indicated in brackets.

Strain	Selective agent	Specific growth rate, $\text{g l}^{-1}\text{h}^{-1}$	Ethanol g l^{-1}	Ethanol productivity $\text{g l}^{-1}\text{h}^{-1}$	Specific ethanol productivity $\text{g g}^{-1}\text{biomass h}^{-1}$	Ethanol yield g g^{-1} of consumed glucose
AS400	-	0.133±0.003	80.3±1.5 (84.6±1.0)	4.46±0.08 (3.53±0.03)	1.86±0.03	0.402±0.007 (0.423±0.005)
AS400-567	oxythiamine	0.133±0.004	85.2±1.6 (90.5±0.9)	4.73±0.09 (3.77±0.04)	1.97±0.04	0.426±0.008 (0.453±0.004)
AS400-543	trehalose	0.083±0.002	84.3±1.6 (88.8±0.9)	4.68±0.09 (3.70±0.03)	2.75±0.06	0.422±0.008 (0.444±0.005)
AS400-617	bromopyruvate	0.133±0.003	84.1±1.5 (89.7±1.0)	4.67±0.09 (3.74±0.03)	1.95±0.03	0.421±0.007 (0.449±0.005)
AS400-510	glyoxylic acid	0.133±0.004	85.1±1.6 (91.4±0.9)	4.73±0.09 (3.81±0.03)	1.97±0.04	0.426±0.007 (0.457±0.005)
AS400-128	glucosamine	0.117±0.003	84.6±1.7 (89.6±0.9)	4.70±0.09 (3.73±0.04)	2.24±0.04	0.423±0.008 (0.448±0.005)
AS400-510-42	glyoxylic acid, glucosamine	0.133±0.004	86.7±1.7 (93.0±0.9)	4.82±0.09 (3.88±0.03)	2.01±0.04	0.434±0.008 (0.465±0.004)
AS400-510-42-214	glyoxylic acid, glucosamine, bromopyruvate	0.117±0.003	88.4±1.5 (94.8±1.0)	4.91±0.08 (3.95±0.04)	2.34±0.04	0.442±0.008 (0.474±0.005)

(±) - absolute error

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5

CLAIMS

What is claimed is:

10

1. A method of making a *S. cerevisiae* strain with enhanced ethanol producing characteristics comprising,

15

contacting a culture of a parent strain of *S. cerevisiae* with a sufficient amount of a first selection agent selected from the group consisting of oxythiamine, trehalose, bromopyruvate, glyoxylic acid and glucosamine for the selection agent to be toxic to the parent strain;

growing the contacted culture on a plating medium containing a sugar as a carbon source;

20

selecting candidate spontaneous mutant strains of the culture that grow on the plating medium, and

measuring an ethanol producing characteristic of the candidate spontaneous mutant strains to determine whether the candidate strain demonstrates enhanced ethanol production characteristics in comparison to the parent strain and if so, obtaining the *S. cerevisiae* strain with enhanced ethanol producing characteristics.

25

2. The method of claim 1 wherein the obtained spontaneous mutant strain with enhanced ethanol production characteristics is contacted with an amount of a second selection agent selected from the group consisting of oxythiamine, trehalose, bromopyruvate, glyoxylic acid and glucosamine for the selection agent to be toxic to the strain, wherein the second selection agent differs from the first selection agent; and

30

repeating the growing, selecting and measuring acts to obtain a second spontaneous mutant strain having enhanced ethanol production characteristics in comparison to the first obtained spontaneous mutant strain.

3. The method of claim 1 or 2 wherein the first selection agent is oxythiamine.

35

4. The method of claim 1 or 2 wherein the first selection agent is trehalose.

5. The method of claim 1 or 2 wherein the first selection agent is bromopyruvate.

- 5 6. The method of claim 1 or 2 wherein the first selection agent is glyoxylic acid.
7. The method of claim 1 or 2 wherein the first selection agent is glucosamine.
8. The method of any of claims 1-7 wherein the enhanced ethanol producing characteristic is at least one of: increased ethanol production measured as wt ethanol produced per volume, increased ethanol productivity measured as wt ethanol produced per
10 volume per unit time, increased ethanol yield defined by wt ethanol produced per wt sugar fed to the strain, and increased specific ethanol productivity defined by wt ethanol produced per weight *S. cerevisiae* biomass per unit time.
9. A *S. cerevisiae* strain with enhanced ethanol producing characteristics made by the method of any of claims 1 -8.
- 15 10. The *S. cerevisiae* strain of claim 9 wherein the parent strain is an industrial ethanol production strain.
11. The *S. cerevisiae* strain of claim 9 selected from the group consisting of AS400-567, AS400-543, AS400-617, AS400-510, AS400-128, AS400-510-42, and AS400-42-214.
- 20 12. A *S. cerevisiae* strain derived from use of the *S. cerevisiae* strain according to any of claims 9-11.
13. A method of producing ethanol comprising fermenting a sugar with a *S. cerevisiae* strain according to any of claims 9-12 under conditions suitable for the production of ethanol.
- 25 14. The method of claim 13 further comprising separating the ethanol produced by the fermentation.
15. A method of making a *S. cerevisiae* strain with enhanced ethanol producing characteristics comprising,
 contacting a culture of a parent strain of *S. cerevisiae* with a sufficient amount of a
30 first selection agent selected from the group consisting of oxythiamine, trehalose, glyoxylic acid and glucosamine for the selection agent to be toxic to the parent strain;

5 growing the contacted culture on a plating medium containing a sugar as a carbon source;

 selecting candidate spontaneous mutant strains of the culture that grow on the plating medium, and

 measuring an ethanol producing characteristic of the candidate spontaneous mutant strains to determine whether the candidate strain demonstrates enhanced ethanol
10 production characteristics in comparison to the parent strain and if so, obtaining the *S. cerevisiae* strain with enhanced ethanol producing characteristics.

16. The method of claim 15 wherein the first selection agent is oxythiamine.

17. The method of claim 15 wherein the first selection agent is trehalose.

15 18. The method of claim 15 wherein the first selection agent is glyoxylic acid.

19. The method of claim 15 wherein the first selection agent is glucosamine.

20. The method of claim 15 wherein the obtained spontaneous mutant strain with enhanced ethanol production characteristics is contacted with an amount of a second selection agent selected from the group consisting of oxythiamine, trehalose, glyoxylic
20 acid and glucosamine for the selection agent to be toxic to the strain, wherein the second selection agent differs from the first selection agent; and

 repeating the growing, selecting and measuring acts to obtain a second spontaneous mutant strain having enhanced ethanol production characteristics in comparison to the first obtained spontaneous mutant strain.

25

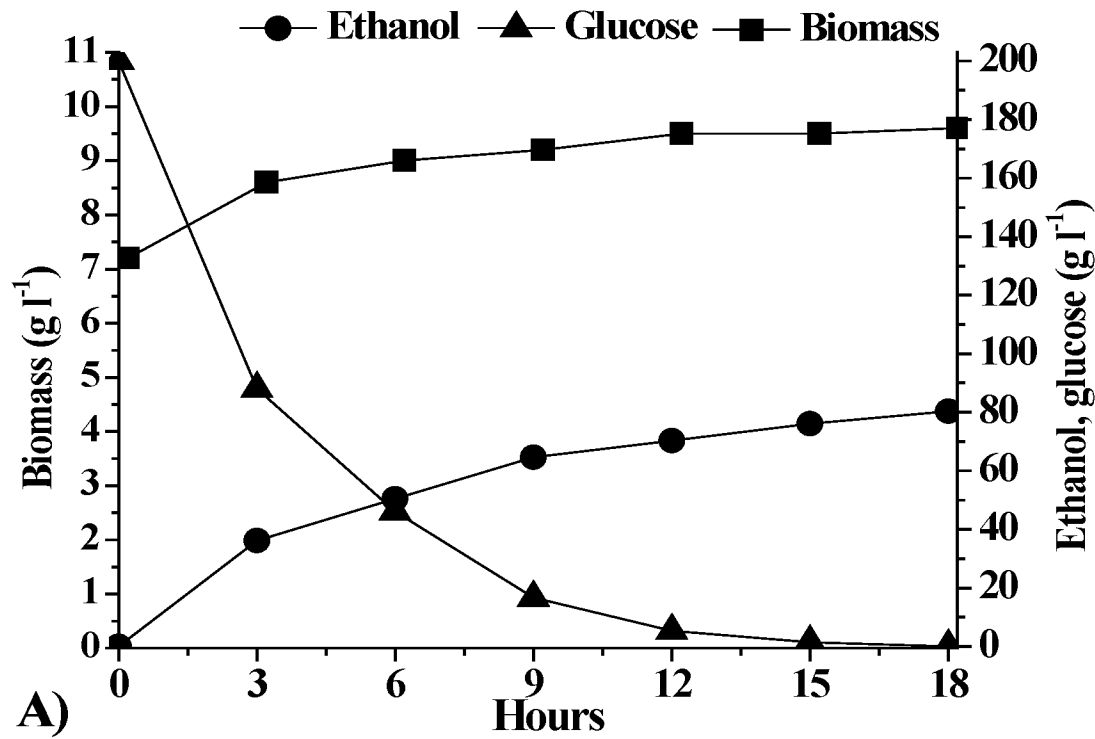


FIG. 1: AS400 fermentation

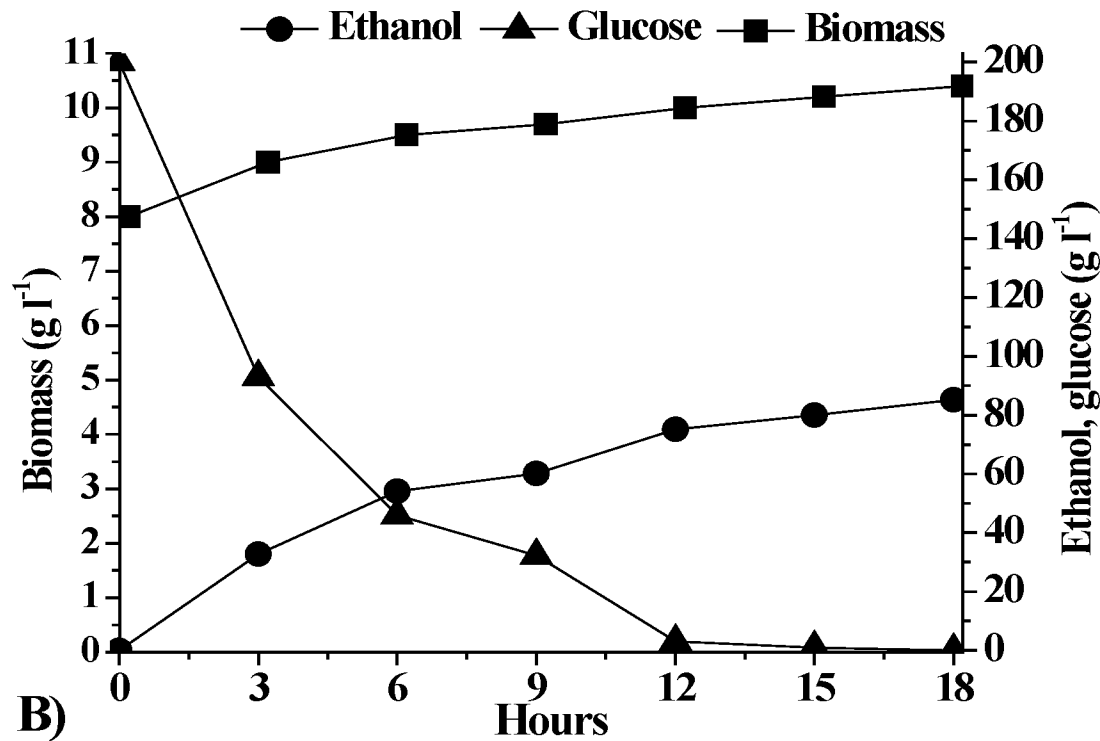


FIG. 2: AS400-567 fermentation

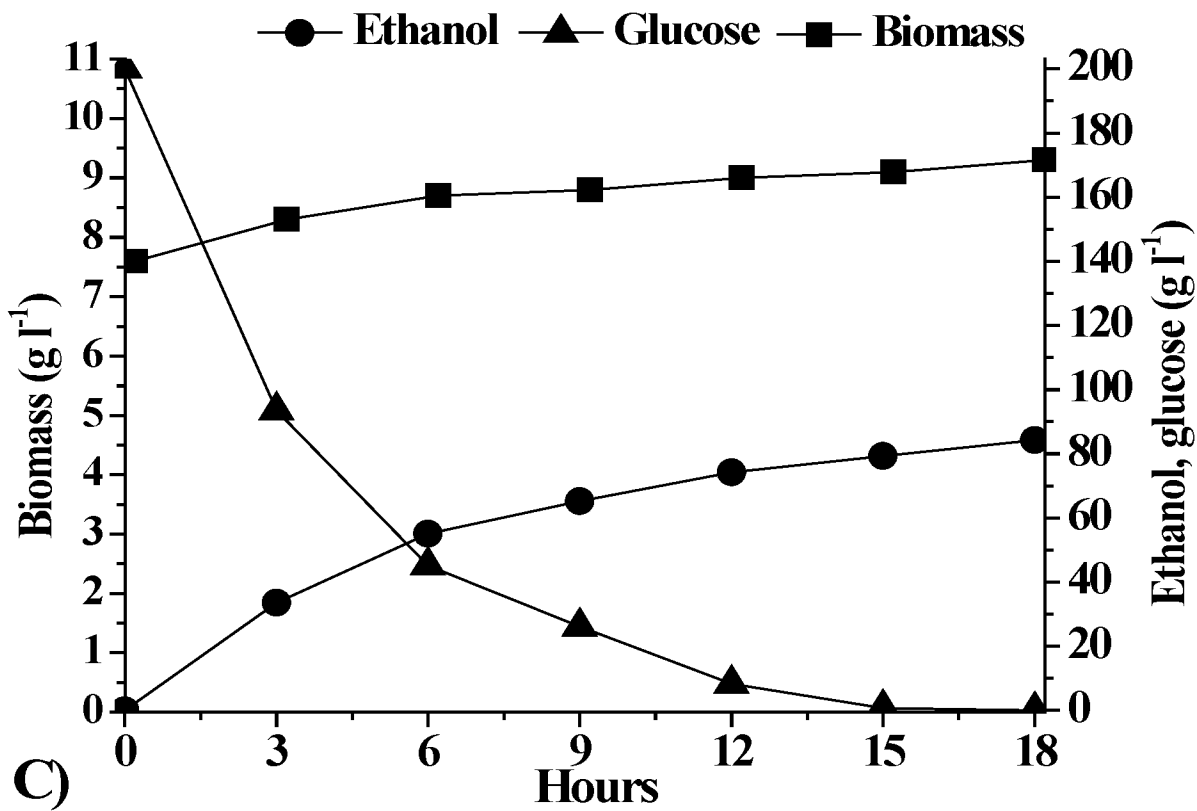


FIG. 3: AS400-543 fermentation

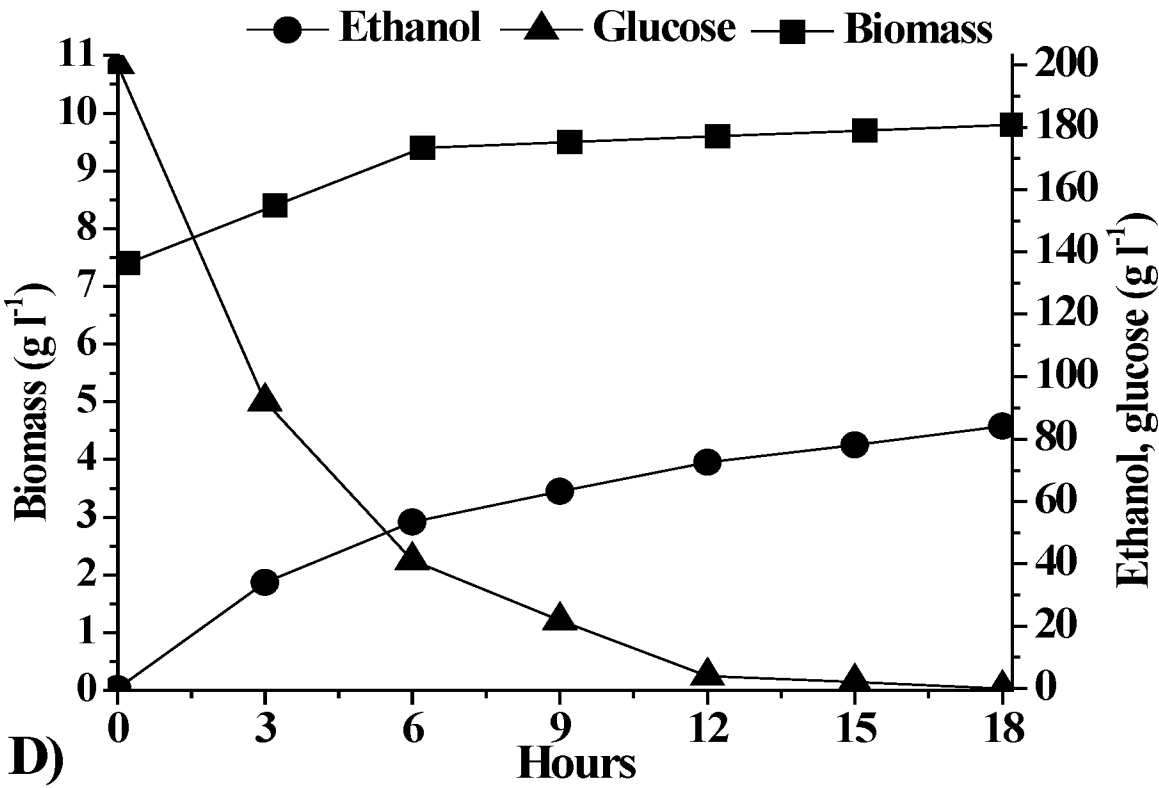


FIG. 4: AS400-617 fermentation

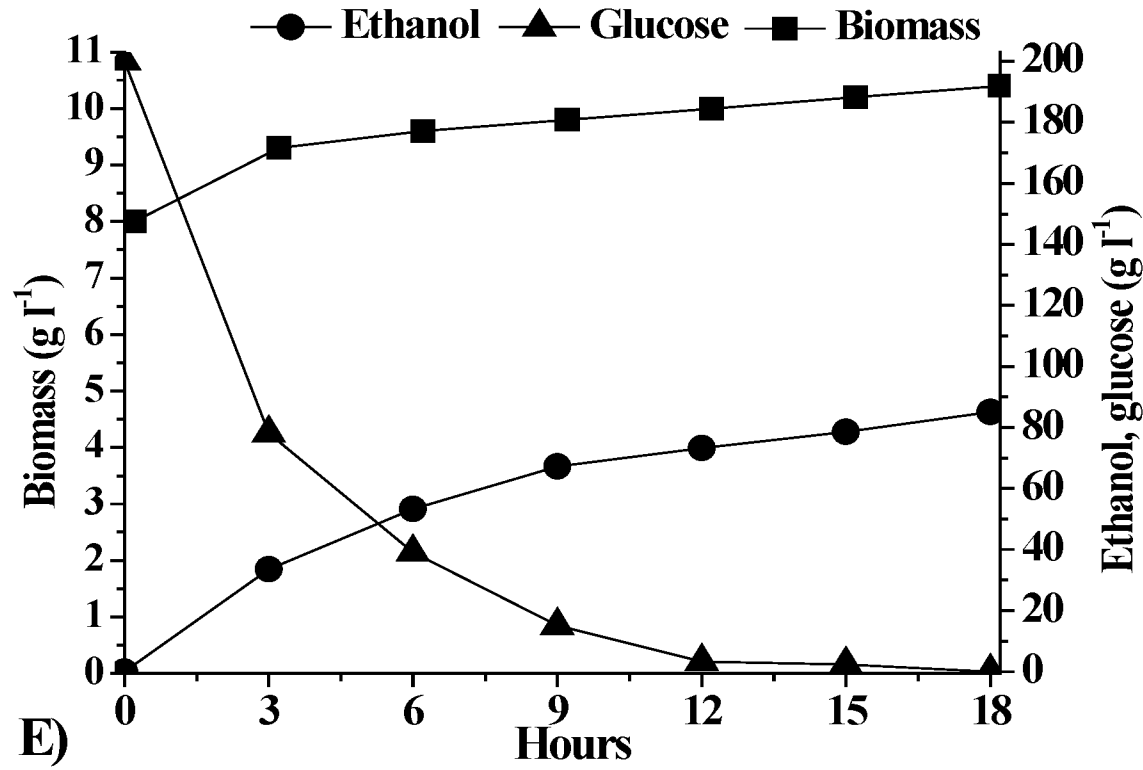
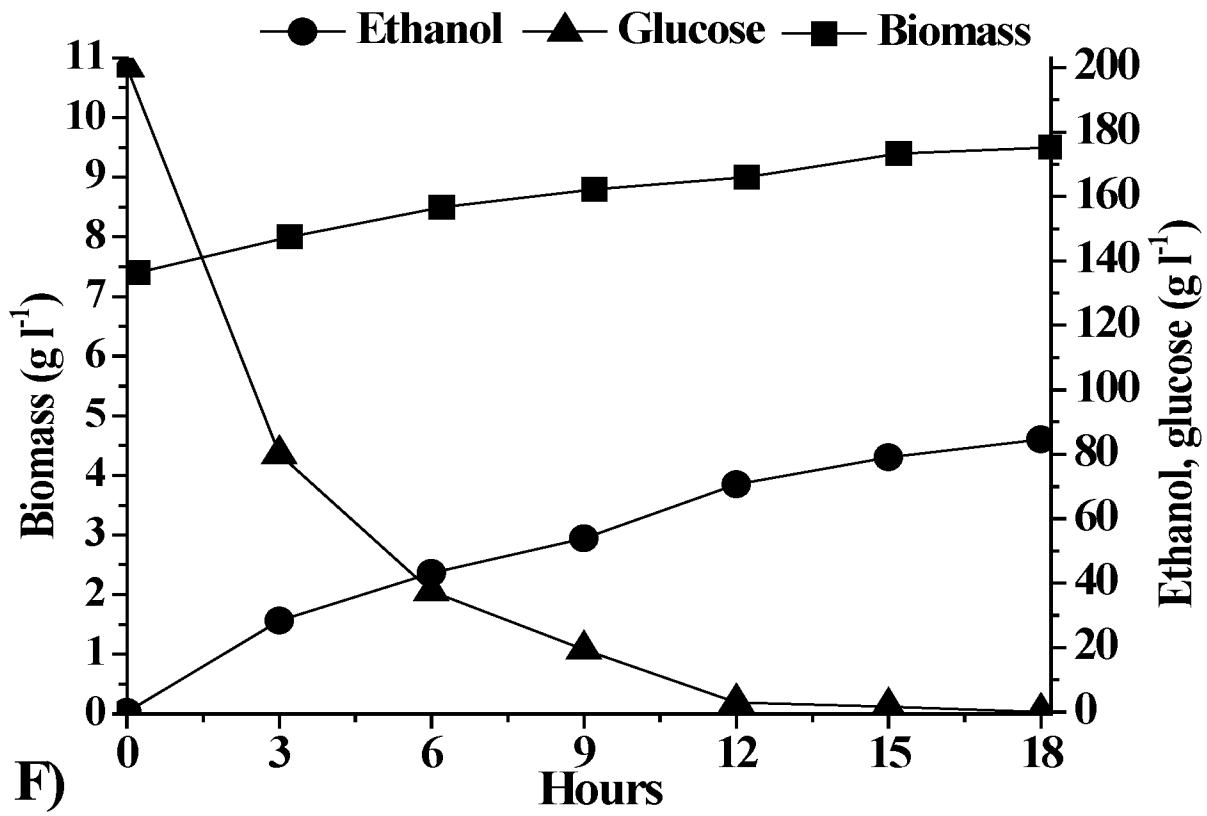


FIG. 5: AS400-510 fermentation



5 FIG. 6: AS400-128 fermentation

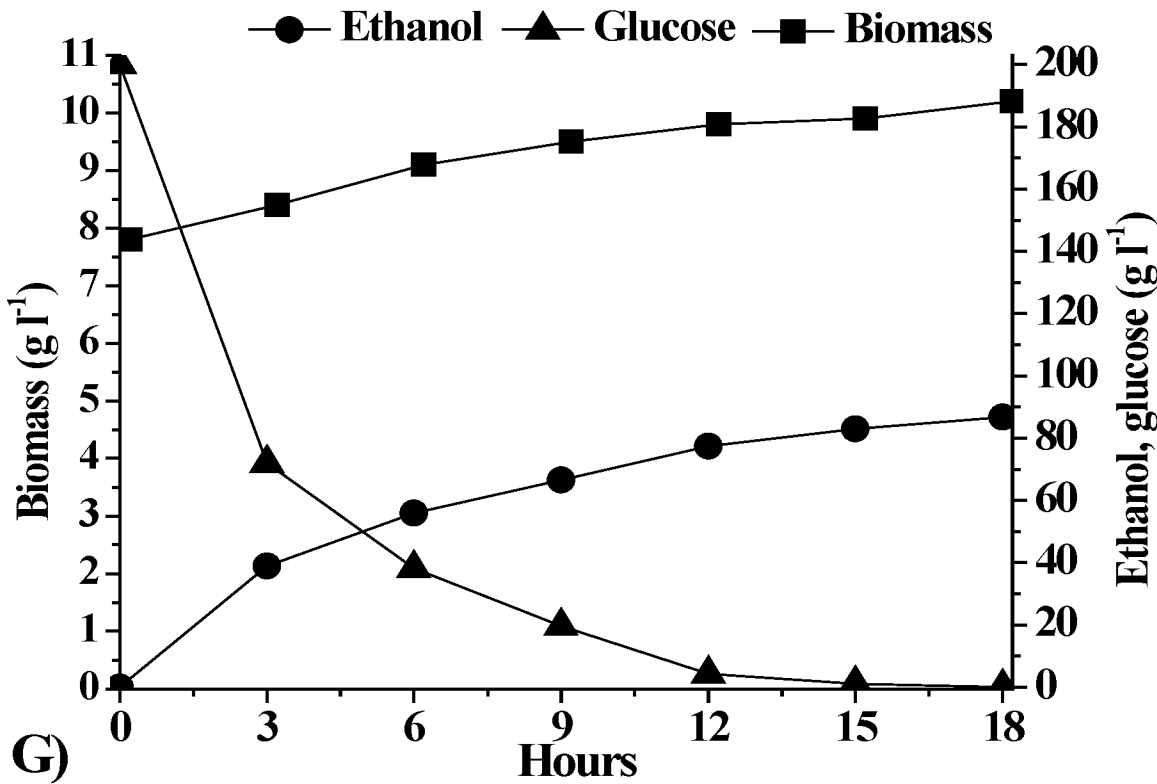
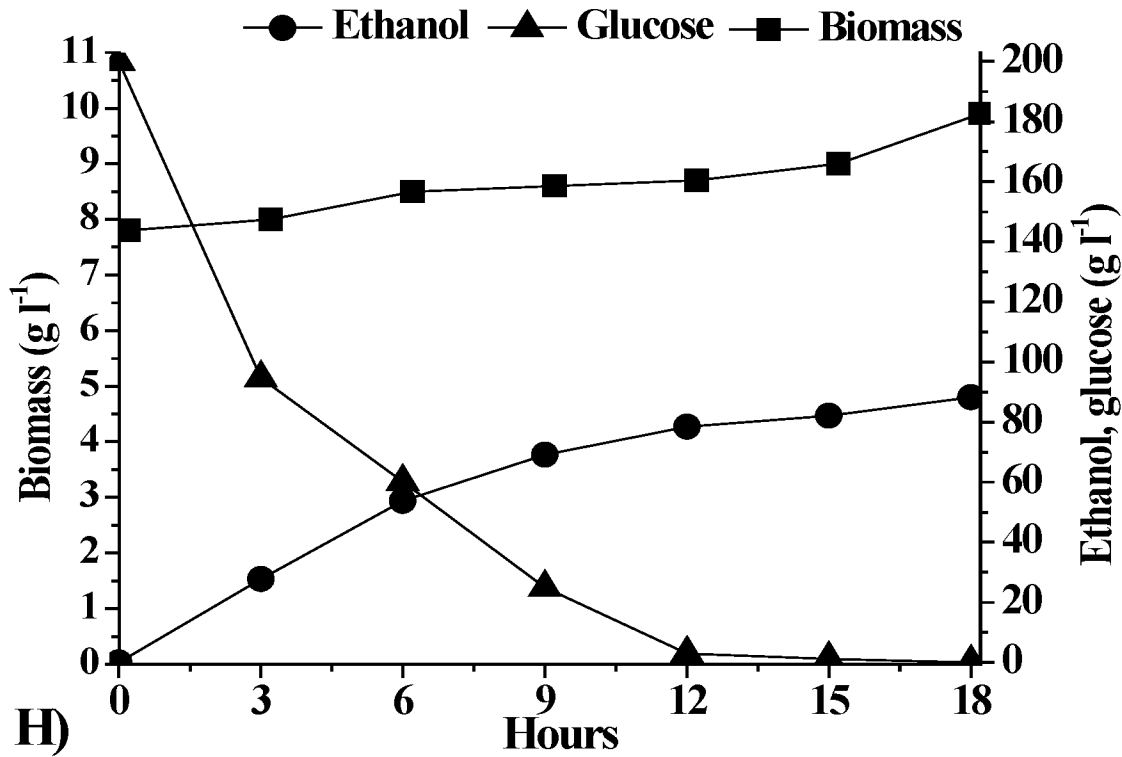


FIG. 7: AS400-510-42 fermentation



5 FIG. 8: AS400-510-42-214 fermentation

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/24910

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12M 1/34, C12N 1/16, C12N 5/04, C12N 15/01, C12P 7/06, C12Q 1/04, C12Q 1/54 (2016.01);

CPC - C12M 1/34, C12N 1/16, C12N 5/04, C12N 15/01, C12N 2830/704, C12P 7/06, C12Q 1/04, C12Q 1/54

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)- C12M 1/34, C12N 1/16, C12N 5/04, C12N 15/01, C12P 7/06, C12Q 1/04, C12Q 1/54 (2016.01);

CPC- C12M 1/34, C12N 1/16, C12N 5/04, C12N 15/01, C12N 2830/704, C12P 7/06, C12Q 1/04, C12Q 1/54

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC- 435/14, 435/34, 435/41, 435/161, 435/255.2, 435/254.21, 435/255.7, 435/285.1, 435/410, 435/440;

Patents and NPL (classification, keyword; search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Pub West (US EP JP WO), Pat Base (AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO), Google Patent, Google Scholar, Free Patents Online; search terms: saccharomyce, cerevisiae, yeast, brewer, ethanol, bioethanol, C2, parent, mutate, mutation, selective, oxythiamine, trehalose, bromopyruvate, pyruvate, glycoxylic, glucosamine...

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0306083 A1 (MUCHA) 15 December 2011 (15.12.2011), para [0008]-[0011], [0042], [0071], [0082], [0086], [0090], [0114], [0124], [0151], [0184]	1-7, 15-20
Y	VAN DIJCK et al. "Differential Importance of Trehalose in Stress Resistance in Fermenting and Nonfermenting Saccharomyces cerevisiae Cells." Applied and Environmental Microbiology [online], January 1995 [Retrieved on 2016-05-31], pp. 109-115, Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC167267/ >, pg 112, col 2, para 3 to pg 113, col 1, para 1; pg 114, col 1, para 2	1-7, 15-20
Y	US 2003/0162164 A1 (BOCHNER et al.) 28 August 2003 (28.08.2003), para [0007], [0049]-[0051], [0094], [0334], [0347], [0366]	3, 5-7, 16, 18, and 19
Y	US 2013/0149760 A1 (FORRESTER et al.) 13 June 2013 (13.06.2013), para [0009]-[0066]	1-7, 15-20
Y	US 2013/0137181 A1 (CHOI et al.) 30 May 2013 (30.05.2013), para [0014]-[0115]	1-7, 15-20

☐ 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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

28 May 2016 (28.05.2016)

Date of mailing of the international search report

05 JUL 2016

Name and mailing address of the ISA/US

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Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/24910

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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.: 8-14
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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.