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(73) Patenthaver: **UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC., 630 Boyd Graduate Studies Research Center, Athens, GA 30602-7411, USA**

(72) Opfinder: **EITEMAN, Mark, A., 1260 Willow Ridge, Athens, GA 30602, USA**
ALTMAN, Elliot, 1051 Summit Oaks Drive, Watkinsville, GA 30677, USA

(74) Fuldmægtig i Danmark: **RWS Group, Europa House, Chiltern Park, Chiltern Hill, Chalfont St Peter, Bucks SL9 9FG, Storbritannien**

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DESCRIPTION

BACKGROUND

[0001] Over the last ten years many reports have provided compelling evidence for a competitively priced bio-based products industry that will replace much of the petrochemical industry. In order to reduce U.S. reliance on foreign oil, there is an increasing interest in generating commodity and fine chemicals from widely available U.S. renewable resources through fermentation. In the last five years, billions of dollars have been invested in commercializing the microbial production of several chemicals, including lactic acid, 1,3-propanediol and 3-hydroxypropanoic acid. In concert with this increased interest in crop-derived biochemicals, molecular biology has become a standard tool which engineers and applied scientists use routinely to aid the rational and targeted alteration of metabolism.

[0002] The efficient use of agricultural biomass for the production of any biochemical, however, is problematic. Technical challenges to be overcome in order for bio-based industrial products to be cost-competitive include finding new technology and reducing the cost of technology for converting biomass into desired bio-based industrial products. Research resulting in cost-effective technology to overcome the recalcitrance of cellulosic biomass would allow biorefineries to produce fuels and bulk chemicals on a very large scale.

[0003] The untapped sources of biomass are largely lignocellulosic in nature. One promising use of lignocellulose for liquid fuel is in the microbial production of ethanol. Unfortunately, when broken down into constituents, a very complex mixture remains. This mixture contains sugars which individually but not collectively are suitable for fermentation, and the mixture also contains inhibitors.

[0004] Because the unit value of chemical products derived from biomass (e.g., ethanol) is generally low while the potential market is large, the economic viability of such processes depends on the yield and productivity. Yield is the quantity of product formed per mass of material input, while productivity is the rate at which the product is generated. Achieving high yield demands that all biomass components be converted, while high productivity requires that the complex conversions be accomplished quickly.

[0005] The conversion of lignocellulosic biomass to any useful fermentation product follows a series of general process operations which include identifying the biomass, harvesting it, various separation/extraction steps, pretreatments, conversion and subsequent purification steps. Although the particular substrate and chemical product determine the details of each operation, the complex structure of biomass invariably necessitates that its components be broken down by various depolymerization strategies.

[0006] Fermentation is the biological process in which sugar substrates, such as glucose and xylose, are converted into fermentation products, such as ethanol. While the term fermentation is usually reserved for anaerobic processes, analogous microbial processes similarly convert sugar substrates into products under a controlled aerobic environment or under conditions of partial oxygenation. One major limitation is that, regardless of a microorganism's ability to metabolize multiple substrates, a single substrate persistently remains the preferred substrate and the consumption of the sugars is asynchronous. Invariably, a multitude of substrates remains long after the preferred substrate has been metabolized. Numerous attempts to engineer microorganisms capable of equally metabolizing more than one substrate have been made (Dien et al., 2002, J. Industr. Micro. 29:221; Sedlak et al., 2003, Enzyme Micro. Technol. 33:19; Chandrakant and Bisaria, 2000, Appl. Micro. Biotechnol. 53:301; Kuyper et al., 2005, FEMS Yeast Res. 5:925; Zhang et al., 1995, Science 267:240). However, none has prevented the polyauxic behavior of linearly metabolizing one sugar at a time when metabolizing sugar mixtures (i.e., first glucose consumption, then xylose, etc.). Moreover, a process for the simultaneous conversion of xylose and glucose sugar mixtures into products by fermentation is described in Eiteman et al. (2008), Journal of Biological Engineering, 2(3):1754-1611. The described process simultaneously uses two substrate-selective strains of *Escherichia coli,* one which is unable to consume glucose and one which is unable to consume xylose. Eiteman et al. has been published on February 27, 2008 and thus between the priority date and the filing date of the present patent.

[0007] A single microorganism alone is unable to convert multiple sugars simultaneously. Instead, any given microorganism has a complex regulatory network which forces sugars to be metabolized sequentially. This sequential nature invariably reduces the overall rate of a fermentation process to generate the desired product.

[0008] Hydrolysis of the lignocellulosic biomass releases variety of sugars, including hexoses (e.g., glucose, mannose), pentoses (e.g., xylose, arabinose), and oligosaccharides, that are released by the hydrolysis of lignocellulosic biomass, and no single microorganism is capable of fermenting all these sugars. Many ethanologenic microbes, including yeast, prefer to use glucose as a substrate. Even when yeast cells are modified genetically to use xylose, they ferment all glucose before switching to the much slower xylose fermentation. Conversion rates can vary greatly depending on such factors as the type of sugar substrate being fermented, environmental conditions (e.g., pH, temperature), and the concentrations of certain products from other metabolic pathways.

[0009] The fraction of pentose sugars which compose biomass can significant; for example, 12% pentose sugars have been reported for *Pinus* spp. and 26% for *Populus* spp. (Saddler and Mackie, 1990, Biomass 22:293). Acid/enzymatic hydrolysis of agricultural materials also generates a significant fraction of pentoses. For example, hydrolysis of peanut hulls results in a mixture containing 44% pentoses (Chandrakant and Bisaria, 2000, Appl. Micro. Biotechnol. 53:301). In order to achieve high yields and productivities, both pentose and hexose fractions must be fully and efficiently utilized. Complicating the matter is the fact that hydrolysis also leads to the formation of acetic acid, which is a known inhibitor to any of the microorganisms that might be used to ferment these sugars into products such as ethanol.

[0010] The efficient conversion of both pentoses and hexoses is a significant hurdle to the economic utilization of biomass hydrolysates for the generation of any fermentation product. The central problem is that either the microorganism used to metabolize the sugars in the mixture consumes the sugar constituents sequentially (e.g., first glucose and then xylose) or the organism is unable to utilize the pentose at all (as is the case with the yeast, *Saccharomyces cerevisiae)*. In a recent comprehensive review Zaldivar et al. succinctly conclude "the lack of a microorganism able to ferment efficiently all sugars released by hydrolysis from lignocellulosic materials has been one of the main factors preventing utilization of lignocellulose" (Zaldivar et al., 2001, Appl. Microbiol. Biotechnol. 56:17).

[0011] Current strategies have focused on the development of a single organism engineered to metabolize both hexoses and pentoses, a single organism that "can do it all." For example, the common yeast *Saccharomyces cerevisiae,* the most widely used organism for ethanol production from starch-based crops, has been genetically modified to metabolize xylose as well as its native substrate glucose. Genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase fused to glycolytic promoters have been successfully integrated into the yeast chromosome (Ho et al., 1998, Appl. Env. Micro. 64:1852.; Sedlak et al., 2003, Enzyme Micro. Technol. 33:19.). In another study, *S. cerevisiae* genetically engineered to contain genes to metabolize xylose still consumed less than 25% of the xylose when glucose was depleted (Sedlak et al., 2003, Enzyme Micro. Technol. 33:19.). Even when xylose isomerase activity was added to *S. cerevisiae* to convert xylose to glucose extracellularly, 75% of the xylose still remained after the glucose was completely consumed (Chandrakant and Bisaria, 2000, Appl. Micro. Biotechnol. 53:301.).

[0012] Bacteria are also frequently used for fermentation processes, but are unable to efficiently metabolize sugar mixtures. In many bacteria, the metabolism of glucose prevents efficient xylose consumption and as a result many researchers have attempted to improve the efficiency of xylose consumption. Introducing a mutation into the *ptsG* gene of *Escherichia coli* can reduce glucose-mediated repression of xylose consumption (Dien et al., 2002, J. Industr. Micro. 29:221.). For example, in batch culture with the ethanologenic *E. coli* strain K011 grown on hemicellulose hydrolysate, for example, only 11% of the xylose was consumed after 24 h, while 80% of the glucose was consumed (Barbosa et al., 1992, Appl. Env. Micro. 58:1382.). Removal of the *ptsG* improved xylose consumption in the presence of glucose, but still 40% of the xylose remained when the glucose is depleted (Dien et al., 2002, J. Industr. Micro. 29:221.).

[0013] Approaches using "evolutionary engineering" have also significantly improved the rate of xylose consumption (Kuyper et al., 2005, FEMS Yeast Res. 5:925), but have not prevented the diauxic behavior when using sugar mixtures (i.e., first glucose consumption, then xylose). Likewise, introduction of genes involved in the xylose metabolism pathway into *Zymomonas mobilis* does not prevent it from consuming xylose much more slowly than glucose (Zhang et al., 1995, Science 267:240). Because both sugars are not consumed effectively in any of these single-organism processes, the productivity of the process is suboptimal.

[0014] Strategies which require a single organism to convert xylose and glucose suffer from several limitations. First, as noted above, the consumption of the sugars is asynchronous. Despite the presence of the genetic apparatus to consume both sugars, glucose remains the preferred substrate, and xylose invariably remains when glucose has been consumed. This asynchronicity particularly influences a single microorganism's ability to cope well with a real hydrolysate having a temporally varying concentration of each sugar. Faced with a time-varying stream, yet using a single organism which has a limited ability to adjust its ratio of glucose and xylose consumption rates, the process will invariably lead to one of the sugars not being effectively consumed. It is not currently possible for one organism to "adjust" its rate of consumption to two substrates in order to match the concentrations encountered in a real process.

[0015] A second shortcoming is that a single microorganism strain, which has been engineered to consume both glucose and xylose, tends to be unstable. A study using a chemostat demonstrated that the presence of both sugars caused an increase over time in the by-product (and inhibitor) acetic acid, which ultimately led to a 20% decrease in ethanol yield (Dumsday et al., 1999, J. Indust. Micro. Biotechnol. 23:701).

[0016] This highlights another complication: hydrolysis of real biomass typically generates compounds which inhibit the subsequent conversion of sugars by fermentation. For example, xylose is acetylated in lignocellulose (Timmel, 1967, Wood Sci. Technol. 1:45; Chesson et al., 1993, J. Sci. Food Agric. 34:1330), and therefore acetic acid is an unavoidable product of hemicellulose depolymerization. Although the inhibitory effect of acetic acid depends on the strain and process, an acetic acid concentration of only 0.08% has been demonstrated to inhibit a subsequent fermentation to generate ethanol (van Zyl et al., 1991, Enzyme Micro. Technol. 13:82). Generally acetic acid inhibits xylose conversion more than it affects glucose conversion. In *S. cerevisiae* engineered to metabolize xylose, for example, acetic acid reduced ethanol yield from xylose by 50% (Helle et al., 2003, Enzyme Microbial Technol. 33:786). Acetic acid itself, and not merely the pH, causes the inhibition. Therefore, base neutralization traditionally applied to acid treated lignocellulosic hydrolysates does not eliminate the inhibitory affects of acetic acid. Previous approaches to convert this mixture of sugars and inhibitors have not been able to achieve the rate of conversion necessary to make the process economically viable. A novel approach that successfully overcomes the inability to convert a mixture of sugars and inhibitors and increases the yields of fermentation product per amount of biomass would represent a significant and long awaited advance in the field.

SUMMARY

[0017] The present invention is defined by the claims. In one aspect, the invention provides a method for producing a biochemical as defined by the claims that includes concurrently contacting a lignocellulosic hydrolysate, with a plurality of sugar-selective cells under conditions to allow the plurality of sugar-selective cells to produce the biochemical. The method optionally includes the additional steps of hydrolyzing a lignocellulosic biomass to produce the hydrolyzed lignocellulosic biomass, and/or purifying the resulting biochemical product. Exemplary biochemicals that can be produced using the method of the invention include ethanol, butanol, succinate, lactate, fumarate, pyruvate, butyric acid and acetone.

[0018] The plurality of sugar-selective cells is in accordance with the present invention as defined in the claims. The plurality of sugar-selective cells being also described herein may include at least one hexose-selective cell and at least one pentose-selective cell. The plurality of sugar-selective cells described herein may include first and second sugar-selective cells. The first sugar-selective cell metabolizes a first sugar that cannot be metabolized by the second sugar-selective cell. The second sugar-selective cell metabolizes a second sugar that cannot be metabolized by the first sugar-selective cell. These first and second sugars are different, and may be selected from the group consisting of glucose, xylose, arabinose, galactose, or mannose. Moreover, at least one of the sugar-selective cells may not metabolize at least two of those sugars, or at least three of those sugars. At least one of the sugar-selective cells may metabolize only one sugar that is independently selected from the group consisting of glucose, xylose, arabinose, galactose, and mannose.

[0019] The sugar selective cells described herein may be single-celled microorganisms, such as yeast or bacteria. Cells suitable for use in the method described herein include cells of *Escherichia coli, Zymomonas mobilis, Corynebacterium glutamicum,* Lactic Acid Bacteria, *Saccharomyces cerevisiae, Pichia stipitis* and *Ambrosioryma monospora.* The plurality of cells may include a first *E. coli* cell wherein at least one gene involved in glucose metabolism has been modified or deleted so as to prevent the first *E. coli* cell from consuming glucose; and a second *E. coli* cell wherein at least one gene involved in xylose metabolism has been modified or deleted so as to prevent the second *E. coli* cell from consuming xylose.

[0020] When the method described herein is used to produce ethanol, the plurality of sugar-selective cells may include at least one cell that has been genetically engineered for enhanced ethanol production.

[0021] Production of the biochemical can also optionally be enhanced when at least one of plurality of sugar-selective cells has been genetically engineered to express or overexpress at least one enzyme selected from the group consisting of an alcohol dehydrogenase enzyme and a pyruvate decarboxylase enzyme.

[0022] Some methods described hereinmake use of an inhibitor-selective cell to metabolize one or more inhibitors, such as acetic acid, furfural and hydroxymethyl furfural (HMF), commonly found in lignocellulosic hydrolysates. The lignocellulosic hydrolysate is contacted with at least one inhibitor-selective cell under conditions to allow the inhibitor-selective cell to metabolize the inhibitor. The inhibitor-selective cell may convert the inhibitor into the desired biochemical. Contact of the inhibitor-selective cell with the lignocellulosic hydrolysate can occur prior to contacting the hydrolysate with the plurality of sugar-selective cells, as part of a two stage process, or concurrent with contacting the hydrolysate with the plurality of sugar-selective cells, as part of a single stage process.

[0023] The inhibitor-selective cell described herein may be a bacteria or yeast, such as an *E. coli* cell, wherein at least one gene involved in glucose metabolism has been modified or deleted so as to prevent the cell from consuming glucose, and at least one gene involved in xylose metabolism has been modified or deleted so as to prevent the cell from consuming xylose. The inhibitor-selective cell may have been genetically engineered such that it cannot metabolize any sugar selected from the group consisting of glucose, xylose, arabinose, galactose and mannose.

[0024] More generally, also described herein is a method for producing a biochemical that includes concurrently contacting an organic material with a plurality of substrate-selective cells under conditions to allow the plurality of substrate-selective cells to produce the biochemical. The substrates for which the cells are selective can include any carbon substrate, including but not limited to sugars, inhibitors, hydrocarbons and the like. In an example, the plurality of substrate-selective cells includes at least one sugar-selective cell and at least one inhibitor-selective cell.

[0025] Also described herein is a method for removing acetate from a mixture, such as a lignocellulosic hydrolysate, that includes acetate as well as one or more sugars, such as glucose and xylose. The mixture is contacted with an acetate-selective cell, such as an *E. coli* cell, under conditions to allow the acetate-selective cell to consume acetate. At least one gene involved in glucose metabolism, as well as at least one gene involved in xylose metabolism, may have been modified or deleted from the acetate-selective cell so as to prevent the cell from consuming glucose and xylose. The acetate-selective cell may have been genetically engineered such that it cannot metabolize any sugar selected from the group consisting of glucose, xylose, arabinose, galactose and mannose. The acetate-selective cell may produce a biochemical selected from the group consisting of ethanol, butanol, succinate, lactate, fumarate, pyruvate, butyric acid and acetone.

[0026] Also described herein is a method for converting a lignocellulosic hydrolysate into a biochemical using a two stage process. In a first stage, a lignocellulosic hydrolysate comprising at least one inhibitor and a plurality of sugars is contacted with at least one microorganism that can only consume an inhibitor, to yield a detoxified hydrolysate. In the second stage, the detoxified hydrolysate is contacted with a plurality of microorganisms, wherein each microorganism independently consumes only one sugar in the hydrolysate, under conditions to allow the plurality of microorganisms to produce the biochemical.

[0027] It should be understood that not only the methods are described herein, but also, without limitation, the substrate-selective cells, and compositions including such substrate-selective cells, are described herein for use in the methods described herein. For example, the present invention as defined by the claims includes an acetate-selective *E. coli* cell, in which at least one gene involved in glucose metabolism has been modified or deleted so as to prevent the cell from consuming glucose, and a modification or deletion of at least one gene involved in xylose metabolism so as to prevent the cell from consuming xylose. The acetate-selective cell may have been genetically engineered such that it cannot metabolize any sugar selected from the group consisting of glucose, xylose, arabinose, galactose and mannose.

[0028] Also described herein is a genetically engineered microorganism that cannot utilize either glucose or xylose as a carbon source, wherein the corresponding wild-type microorganism utilizes both glucose and xylose as a carbon source. Also included is a genetically engineered microorganism that cannot utilize either glucose or arabinose as a carbon source, wherein the corresponding wild-type microorganism utilizes both glucose and arabinose as a carbon source. The microorganism may be an *E. coli, Z. mobilis, C. glutamicum,* Lactic Acid Bacteria, *S. cerevisiae, P. stipitis* or *A. monospora*. Also described herein is a genetically engineered bacterium that cannot utilize either glucose or xylose as a carbon source. Further described herein is a genetically engineered bacterium that cannot utilize either glucose or arabinose as a carbon source. Also included is a genetically engineered bacterium that cannot utilize either glucose or galactose as a carbon source. The bacterium may be an *E. coli, Z. mobilis, C. glutamicum,* or Lactic Acid Bacteria.

[0029] Yet other exemplary compounds described herein include *Pichia stipitis* that cannot utilize either glucose or xylose as a carbon source; *Pichia stipitis* that cannot utilize either glucose or arabinose as a carbon source; *Ambrosiozyma monospora* that cannot utilize either glucose or xylose as a carbon source; and *Ambrosiozyma monospora* that cannot utilize either glucose or arabinose as a carbon source.

[0030] The invention further includes a composition as defined by the claims comprising first and second sugar-selective cells, wherein the first sugar-selective cell metabolizes a first sugar that cannot be metabolized by the second sugar-selective cell, and the second sugar-selective cell metabolizes a second sugar that cannot be metabolized by the first sugar-selective cell, and wherein the first and second sugars are independently selected from the group consisting of glucose, xylose, arabinose, galactose, and mannose. For example, at least one of the sugar-selective cells can metabolize only one sugar independently selected from the group consisting of glucose, xylose, arabinose, galactose, and mannose. Optionally the composition further includes an inhibitor-selective cell, such as an inhibitor-selective cell that has been genetically modified such that it cannot metabolize any sugar selected from the group consisting of glucose, xylose, arabinose, galactose and mannose. The sugar-selective cells include in accordance with the invention *E. coli* and *S. cerevisiae*. Optionally, at least one of the sugar-selective cells has been genetically engineered for enhanced ethanol production. Also optionally, at least one of the sugar-selective cells has been genetically engineered to express or overexpress at least one enzyme selected from the group consisting of an alcohol dehydrogenase enzyme and a pyruvate decarboxylase enzyme. The composition optionally further includes a lignocellulosic hydrolysate.

[0031] Also included in the invention is a composition as defined in the claims that includes a plurality of microorganisms of the same species, being in accordance with the claims *E. coli* or *S. cerevisiae,* wherein each microorganism independently utilizes only one sugar, such as glucose, xylose, arabinose, galactose, and mannose, that is present in a lignocellulosic hydrolysate.

[0032] It is to be understood that the terms used herein to describe acids (for example, the term acetate) are not meant to denote any particular ionization state of the acid, and are meant to include both protonated and unprotonated forms of the compound. Thus, the terms acetate and acetic acid refer to the same compound and are used interchangeably.

[0033] The description that follows more particularly exemplifies illustrative embodiments.

BRIEF DESCRIPTION OF THE FIGURES

[0034]

Fig. 1 shows a two-step process to a) consume inhibitor acetic acid and b) to convert xylose and glucose concurrently into a desired product.

Fig. 2 shows batch aerobic fermentation of *Escherichia coli* MG1655 on a mixture of glucose (□) and xylose (Δ). The OD (●) was measured over the course of fermentations.

Fig. 3 shows batch aerobic fermentation of single *Escherichia coli* strains on a mixture of glucose (□) and xylose (Δ). The OD (●) was measured over the course of fermentations inoculated with A) ZSC113 only and B) ALS1008 only.

Fig. 4 shows batch aerobic co-fermentation of *Escherichia coli* strains ZSC113 and ALS1008 on a mixture of glucose (□) and xylose (Δ). The OD (●) was measured over the course of the fermentation.

Fig. 5 shows fed-batch aerobic co-fermentation of *Escherichia coli* strains ZSC113 and ALS1008 using a feed containing a varying mixture of glucose (dotted lines) and xylose (dashed lines). The OD (●), the concentrations of glucose (□) and xylose (Δ), and the fraction of the total cell population which is ZSC113 (▼) were measured over the course of the fermentation.

Fig. 6 shows batch anaerobic fermentation of *Escherichia coli* MG1655 on a mixture of glucose (□) and xylose (Δ). After 7 h (h, hours) of aerobic growth, additional xylose and glucose were added and anaerobic conditions commenced. The concentrations of formate (▲), lactate (■), succinate (○), acetate (◆) and ethanol (◇) were measured over the course of the anaerobic phase.

Fig. 7 shows batch anaerobic fermentation of single *Escherichia coli* strains on a mixture of glucose (□) and xylose (Δ). After 7 h of aerobic growth, xylose (for ZSC113) or glucose (for ALS1008) was added and anaerobic conditions commenced. The concentrations of formate (▲), lactate (■), succinate (○), acetate (◆) and ethanol (◇) were measured over the course of the anaerobic phase previously inoculated with A) ZSC 113 only and B) ALS1008 only.

Fig. 8 shows batch anaerobic co-fermentation of *Escherichia coli* strains ZSC113 and ALS1008 on a mixture of glucose (□) and xylose (Δ). After 7 h of aerobic growth, xylose and glucose were added and anaerobic conditions commenced. The concentrations of formate (▲), lactate (■), succinate (○), acetate (◆) and ethanol (◇) were measured over the course of the anaerobic phase.

Fig. 9 shows batch aerobic fermentation of *Escherichia coli* ALS1060 (knockouts in *ptsG manZ glk xylA*) on a mixture of glucose (□), xylose (Δ) and acetate (▲). The OD (●) was measured over the course of the fermentation time.

Fig. 10 shows batch aerobic-anaerobic process of *Escherichia coli* ALS1073 on a mixture of glucose (■) and xylose (▲). After 8 h of aerobic growth, the culture was switched to anaerobic conditions as indicated. The OD (●) and lactate concentration (◇) were measured over the course of the fermentation.

Fig. 11 shows batch aerobic-anaerobic process of *Escherichia coli* ALS1074 on a mixture of glucose (■) and xylose (▲). After 8 h of aerobic growth, the culture was switched to anaerobic conditions as indicated. The OD (●) and lactate concentration (◇) were measured over the course of the fermentation.

Fig. 12 shows batch aerobic-anaerobic process of two *Escherichia coli* strains ALS1073 and ALS1074 on a mixture of glucose (■) and xylose (▲). After 8 h of aerobic growth, the culture was switched to anaerobic conditions as indicated. The OD (●) and lactate concentration (◇) were measured over the course of the co-fermentation.

DETAILED DESCRIPTION

[0035] The present invention is defined by the claims. Described herein is a novel biological method that permits efficient conversion of a complex organic material to a desired biochemical product. The method described allows multiple sugars in a complex mixture to be consumed concurrently and independently. Each sugar is converted into the desired product at relatively high yield, and the process can adapt to fluctuating sugar concentrations without leading to the accumulation of any one sugar. Moreover, as described further below, the method can readily be extended to remove inhibitory compounds from hydrolysates.

[0036] The complex organic material that serves as the "input" for the method described may be a biomass, such as a lignocellulosic biomass. The term "biomass" can include any plant, vegetation, or waste product that can be used as a fuel or used as an energy source. Cellulosic or lignocellulosic biomass is typically a plant biomass composed of cellulose, hemicellulose and/or lignin, often with smaller amounts of proteins, lipids (fats, waxes and oils) and ash. It should be noted that the terms "cellulosic biomass" and "lignocellulosic biomass" are used interchangeably herein. In some cellulosic biomasses, roughly two-thirds of the dry mass of cellulosic materials is present as cellulose and hemicellulose. Lignin makes up the bulk of the remaining dry mass. Cellulosic biomass feedstocks include agricultural plant wastes (e.g., corn stover, leaves and stalks, cereal straws, sugarcane bagasse), plant wastes from industrial processes (e.g., wood residues, sawdust, paper pulp, wood chips, municipal paper waste) and energy crops grown specifically for fuel production, such as switchgrass. The method described herein can also be practiced using crops and feedstocks such as corn, sugarcane, wheat or barley, and grasses such as switchgrass and hemp. Any organic material that contains a plurality of sugars, whether naturally occurring or synthesized, can serve as the "input" for the method described herein. The organic material, such as the biomass, may be hydrolyzed. The principal monomeric sugars found in the lignocellulosic hydrolysate are glucose, xylose, mannose, galactose and arabinose. These sugars have been found up to the following percentages in various lignocellulosic hydrolysates: arabinose, 17%; galactose, 15%; glucose, 50%; mannose, 36%; xylose, 74%.

[0037] The organic material is converted into the desired biochemical product or products (the "outputs" of the method) by way of a biological process. Efficient conversion of inputs to desired outputs is accomplished by utilizing a plurality of genetically engineered "substrate-selective" cells, each of which has been independently metabolically engineered to result in enhanced consumption of one particular substrate in relation to another, as described in more detail below. Examples of biochemical products include, but are not limited to, ethanol, butanol, succinate, lactate, fumarate, pyruvate, butyric acid and acetone. Other biochemicals that can be produced using the biological method described herein include lysine, gluconate, citrate, malate, and whole-cell yeast biomass. The method described herein may produce ethanol.

[0038] For example, the method described herein encompasses concurrently contacting an organic material, such as a hydrolyzed biomass, with a plurality of substrate-selective cells, under conditions to allow formation of a desired product. The organic material may be mixed with two or more different substrate-selective cells at the same time, in a co-culture, in a single bioreactor. The plurality of substrate-selective cells may include at least one, or at least two, cells that are selective for sugars (sugar-selective cells). The sugar-selective cells may include at least one hexose-selective (HSC), at least one pentose-selective cell (PSC), or a combination thereof. Particularly, the method described herein may concurrently utilize at least one hexose-selective cell and at least one pentose-selective cell.

[0039] Optionally, the method described herein further includes the use of at least one substrate-selective cell that is an inhibitor-selective cell (ISC). The inhibitor-selective cell can be contacted with the hydrolyzed biomass either prior to, or concurrently with, contacting the biomass with the plurality of sugar-selective cells.

[0040] Also described herein is contacting a hydrolyzed biomass with at least one sugar-selective cell and at least one inhibitor-selective cell, under conditions to allow formation of a desired product. Contacting the hydrolyzed biomass with the different substrate-selective cells can occur concurrently or sequentially. If sequential, the hydrolyzed biomass may be first contacted with the inhibitor-selective cell.

[0041] A hexose-selective cell used in the method described herein may be a glucose-selective cell, but can include any other hexose-selective cell such as cells that are selective for glucose, mannose, galactose, allose and fructose. A pentose-selective cell used in the method described herein may be a xylose-selective cell, but can include any other pentose-selective cell such as cells that are selective for xylose, arabinose, ribose, and ribulose.

[0042] A inhibitor-selective cell for use in the method described herein may be an acetate-selective cell (ASC). Acetate consumption in *E. coli* is mediated by acetyl CoA synthase (*acs*), isocitrate lyase (*aceA*), malate synthase (*aceB*) and isocitrate dehydrogenase (*icdA*). Many other organisms *Saccharomyces cerevisiae* and *Pseudomonas spp., Pichia stipitis, Bacillus cereus,* have at least some of these enzymes to degrade acetate however it should be noted that the names of the genes are usually different. For example, in *S. cerevisiae* the gene encoding for isocitrate lyase is named ICL1. An acetate-selective bacterial cell may have deletions in genes involved in the uptake of the all the competing substrates: glucose, mannose, galactose, xylose and arabinose. To that end, the genes *manZ, ptsG, glk, xylA, galK, araA* may be knocked out to yield an acetate-selective cell. As described in more detail below, the inhibitor-selective cell can be contacted with the hydrolyzed biomass either prior to, or concurrent with at least one, or at least two, sugar-selective cells.

[0043] It should be understood that the terms "substrate-selective cell," "sugar-selective cell," "hexose-selective cell," "pentose-selective cell," "inhibitor-selective cell," "glucose-selective cell," "xylose-selective cell," "acetate-selective cell," and the like are not intended to refer to just a single, physical cell, but include many such cells, as in a typical cell culture. Thus, a mixture described herein that contains, for example, one "xylose-selective cell" and one "glucose-selective cell" means a co-culture of xylose-selective cells and glucose-selective cells. Likewise, a mixture that contains two "hexose-selective cells" means a co-culture of first hexose-selective cells (e.g., glucose-selective cells) and second (different) hexose-selective cells (e.g., galactose-selective cells).

[0044] Described herein is not only a method of producing a desired product from a biomass using a plurality of substrate-selective cells, but also the various substrate-selective cells, alone or in combination, as described herein. More particularly, also described herein is a composition that includes a plurality of substrate-selective cells and, optionally, a lignocellulosic biomass or hydrolysate.

Cells

[0045] Cells useful in the method described herein include animal, plant, yeast, protozoan, and bacterial cells. Bacterial cells and yeast cells are in common use for large-scale industrial fermentations. Examples of bacterial cells for use in the method described herein include *Escherichia coli, Zymomonas mobilis, Corynebacterium glutamicum,* Lactic Acid Bacteria such as *Lactobacillus* or *Lactococcus, Pseudomonas spp.* and *Bacillus spp.* such as *B. subtilis* and *B. cereus.* Lactic Acid Bacteria have several characteristics which make them well suited for the biological production of biochemical products. They are natively tolerant of the low pH levels, increased temperatures, and ethanol concentrations of hydrolysates, and are therefore less susceptible than other bacteria to fermentation inhibition. Yeast cells include *Saccharomyces cerevisiae, Pichia stipitis* and *Ambrosiozyma monospora:* While cells of single cell microorganisms such as bacteria, yeast and protozoa are expressly described, the method can also be practiced using animal or plant cell cultures derived from multicellular organisms.

Substrate selectivity

[0046] Metabolic engineering involves the targeted and purposeful alteration (using genetic engineering techniques) of an organism's metabolic pathways to redesign them to utilize different proportions or combinations of substrates, or to produce different proportions or combinations of products. In broad terms, metabolic engineering encompasses genetically overexpressing particular enzymes at selected points in a metabolic pathway, and/or blocking the synthesis of other enzymes, to overcome or circumvent metabolic "bottlenecks." A goal of metabolic engineering is to optimize the rate and conversion of a substrate into a desired product.

[0047] The present disclosure employs a plurality of genetically engineered microorganisms which overcomes the numerous limitations currently encountered in the field when utilizing a single microorganism. Cells useful herein have been metabolically engineered to result in enhanced consumption of one particular substrate in relation to another. At least one metabolic pathway of the cells has been disrupted or altered. In the context described herein, disruption or alteration of a metabolic pathway encompasses disruption in the cellular process for uptake of the substrate from the extracellular environment that reduces or eliminates the ability of the cell to internalize the substrate, as well as a disruption or alteration of an intracellular process involve in the utilization of the substrate. In short, the cells used herein are metabolically engineered to be "substrate-selective."

[0048] A cell is "substrate-selective" when it preferentially utilizes a single organic substrate as a carbon source. Carbohydrates (sugars) represent the most common carbon substrates utilized by bacteria and yeast, but many other organic substrates can be used as carbon sources including hydrocarbons, lipids, proteins, and small organic molecules such as acids, esters and the like. It should be understood that substrate-selective cells described herein include cells that have been engineered to preferentially utilize a particular sugar, inhibitor molecule, hydrocarbon or any other carbon source, without limitation. Such cells are readily identified, designed and selected for use in the method described herein by one of skill in the art based upon the composition of the organic material to be metabolized, and the nature of the desired product.

[0049] One way a cell can be made substrate-selective for a particular substrate is by modifying it, typically through genetically engineering, to reduce or eliminate the cell's ability to metabolize at least one competing substrate. For example, a cell that selectively metabolizes glucose can be made by reducing or eliminating the metabolism of any or all competing substrates such as xylose, galactose, and arabinose. More specifically, a cell that normally consumes both glucose and xylose can be made glucose-selective by reducing or eliminating the cell's ability to metabolize xylose, and xylose-selective by reducing or eliminating the cell's ability to metabolize glucose. Likewise, a cell that normally consumes glucose, xylose and arabinose can be made glucose-selective by reducing or eliminating xylose metabolism, arabinose metabolism, or both; can be made xylose-selective by reducing or eliminating arabinose metabolism, glucose metabolism, or both; and can be made arabinose-selective by reducing or eliminating glucose metabolism, xylose metabolism, or both. More generally, if a cell can metabolize n sugars (where $n > 1$), the cell can be made selective for a particular sugar by reducing or eliminating the metabolism of at least 1 other sugar, up to n-1 other sugars in the most preferred embodiment. A plurality of sugar-selective cells useful in the method described herein thus includes at least a first sugar-selective cell that metabolizes a first sugar that cannot be metabolized by a second sugar-selective cell, and a second sugar-selective cell that metabolizes a second sugar that cannot be metabolized by the first sugar-selective cell.

[0050] These genetic modifications may result in a strain that will only utilize a selected sugar (such as glucose, xylose, arabinose, galactose) or inhibitor (such as acetic acid or a furfural), a strain that can be considered to have "substrate-selective uptake" since it is selective in what compound it is able to consume. For example, by deleting a key gene in the xylose uptake pathway, a strain of bacteria is constructed which is unable to consume xylose, thereby allowing glucose to serve as the specified substrate. Placed in a fermenter containing xylose and glucose, such a strain would leave the xylose unutilized and be completely unaffected by the presence of xylose. Similarly, a strain can be constructed which is unable to consume glucose. Placed in a fermenter with xylose and glucose, such a strain would be unable to utilize the glucose, and thereby consume only xylose.

[0051] Advantageously, a substrate-selective cell functions independently of other (different) substrate-selective cells, as well as independently of other substrates present in the mixture. Placed together in co-fermentation in a fermenter containing glucose and xylose, each strain would be expected to act optimally on just one of these sugars and be unaffected by the presence of the other sugar or the other organism. For example, a glucose specific microorganism could be engineered by eliminating the pathways necessary for metabolism of arabinose, galactose and xylose.

[0052] Additionally, as discussed in more detail below, the metabolic pathways of each strain could be further engineered to optimize yield and productivity of a particular product, such as ethanol.

[0053] A reduction or elimination in the cell's ability to metabolize a particular substrate can be detected in any convenient manner. For example, if a gene has been physically disrupted, e.g., by mutagenesis, deletion, or the like, the disruption can be detected using DNA sequencing or other routine DNA detection procedures. Phenotypically, a reduction or elimination in the cell's ability to metabolize a particular substrate can be detected by comparing the cell's metabolic behavior relative to a mixture of sugars before and after the modification. If the metabolism of at least one sugar is essentially unchanged, but with respect to a second sugar if that sugar is metabolized slower or not at all, one can conclude that there has been a reduction or elimination of the cell's ability to metabolize the second sugar.

[0054] The pathway(s) for metabolizing one or more competing substrates in the substrate-selective cell may be completely disrupted (e.g., via a "knockout" of an essential gene), such that the competing substrate or substrates otherwise metabolized by the cell are not metabolized at a detectable level. A knockout of a competing metabolic pathway can be accomplished by modifying or deleting one or more genes involved in metabolizing the competing substrate, as necessary, so as to prevent the cell from metabolizing the competing substrate.

[0055] Methods of disrupting or altering metabolic pathways in bacteria, plants, and animals to reduce or eliminate a cell's ability to metabolize at least one carbon substrate are routine and well known in the art. Once a molecular target involved in the substrate's metabolism has been identified, disruption of a metabolic pathway can be effected at any level of gene expression (e.g., DNA replication, transcription or translation), or post-translationally. For example, enzymatic function can be inhibited when the enzyme is targeted by a molecular inhibitor, such as an antibody or a small molecule inhibitor. Translation of an RNA message into an enzyme can be disrupted, for example, by introducing a small interfering RNA, a short-hairpin RNA, or a hybridization probe into the bacteria, plant, or animal cell. Transcription of a gene encoding an enzyme can be disrupted, for example, by targeting the gene with a molecular inhibitor or physically altering the gene to prevent or confound gene replication or transcription. Cells can be metabolically engineered through the introduction of polynucleotides, as well as the directed mutagenesis of coding regions. Common gene disruption techniques include mutagenesis, gene deletion or knock-out, and heterologous gene transformation. Such methods are well known in the art; see, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual., Cold Spring Harbor Laboratory Press (1989), and Methods for General and Molecular Bacteriology, (eds. Gerhardt et al.) American Society for Microbiology, chapters 13-14 and 16-18 (1994).

[0056] A particularly useful method for metabolically engineering a substrate-selective cell is to "knock out" an essential gene in the metabolic pathway of a competing substrate. A knock-out technique can be used to render a cell unable to utilize a selected sugar as a carbon source. A bacterial strain that is unable to use glucose as a carbon source can be constructed by knocking out the genes encoding for glucosephosphotransferase (*ptsG*), mannosephosphotransferase (*manZ*) and glucokinase (*glk*) (see Example I). The resulting strain may still be able to metabolize xylose and other sugars, depending on what other functioning metabolic pathways it contains, but not glucose or mannose.

Molecular targets for generating cells that cannot utilize one or more sugars as a carbon source

[0057] The molecular targets of metabolic engineering that will yield a substrate-selective cell for use in the method described herein naturally depend on the nature of the organism, as well as the substrate whose metabolism is to be disrupted.

[0058] Particularly preferred targets for metabolically engineering cells useful in the method described herein are molecules involved in carbohydrate transport and metabolism. A metabolic pathway involved in the metabolism of a selected sugar, for example, can be disrupted by affecting the production or activity of one or more enzymes required, either directly or indirectly, to convert the substrate into the product. Alternatively, knocking out the production of a receptor molecule needed for uptake or transport of the substrate from the extracellular environment also reduces or eliminates a cell's ability to metabolize the substrate.

Constructing bacterial strains that are incapable of utilizing specific sugars

[0059] The phosphotransferase systems of bacteria can translocate a wide range of carbon sources into the cytoplasm of bacteria. The overall process can be represented as phosphoenolpyruvate + carbohydrate → pyruvate + carbohydrate-P where the carbohydrate is phosphorylated and translocated across the cytoplasmic membrane so it can be catabolyzed. There are many phosphotransferase systems which can translocate a wide variety of carbohydrates. While most carbohydrates are translocated by a specific phosphotransferase system (PTS), glucose is unusual in that it can be translocated by any of three different phosphotransferase systems: a glucose-specific glucose PTS that can only translocate glucose, a general glucose PTS that can translocate ribitol, sorbose and other carbohydrates in addition to glucose, and the mannose PTS which can translocate glucose in addition to mannose. Thus to prevent a bacterium from being able to utilize glucose as a carbon source, the glucose-specific, the general glucose, and the mannose phosphotransferase systems must all be knocked out.

[0060] Some carbohydrates must enter the cell via a periplasmic binding protein-dependent (BPD) ABC transporter system where the carbohydrate is translocated across the cytoplasmic membrane and subsequently phosphorylated so it can be catabolyzed. The periplasmic BPD ABC transporter systems all utilize a periplasmic binding protein, an inner or cytoplasmic membrane protein and an ATPase protein to facilitate transport.

[0061] The sugars that comprise lignocellulosic hydrolysates include arabinose, galactose, glucose, mannose, and xylose. The great majority of bacteria can utilize these sugars as their primary carbon source. *Escherichia coli* as well as many of the Lactic Acid Bacteria can utilize all of these sugars. Bacterial strains that are unable to utilize arabinose, galactose, mannose, and xylose as carbon sources can be constructed by eliminating one gene from the respective sugar pathway as indicated in the following table. The exception is glucose: to construct a bacterial strain that is incapable of utilizing glucose, either the *manX, manY* or *manZ* gene must be eliminated in addition to the *glk* and *ptsG* genes.

Examples of pathways for sugar utilization in bacteria

[0062]

Enzyme	**Gene**	**Reaction catalyzed**	**Pathway**
Arabinose utilization			
Arabinose-Binding Protein	araF	Arabinose transport	Arabinose
			Uptake
Arabinose Transport Membrane Protein	araH	Arabinose transport	Arabinose Uptake
Arabinose ATPase Protein	araG	Arabinose transport	Arabinose Uptake
Arabinose Isomerase	araA	Arabinose → Ribulose	Arabinose Catabolism
Ribulokinase	araB	Ribulose → Ribulose-5-P	Arabinose Catabolism
Galactose utilization			
Galactose Binding Protein	mglB	Galactose transport	Galactose Uptake
Galactose Transport Membrane Protein	mglC	Galactose transport	Galactose Uptake
Galactose ATPase Protein	mglA	Galactose transport	Galactose Uptake
Galactokinase	galK	Galactose → Galactose-1-P	Galactose Catabolism
Glucose utilization			
Glucokinase	glk	Glucose → Glucose-6-P	Glucose Uptake
Glucosephosphotransferase Enzyme II	ptsG	Glucose → Glucose-6-P	Glucose Uptake
Mannose PTS Protein IIA(III)	manX	Glucose → Glucose-6-P	Glucose Uptake
Mannosephosphotransferase Enzyme IIB	manZ	Glucose → Glucose-6-P	Glucose Uptake
Mannose utilization			
Mannose PTS Protein IIA(III)	manX	Mannose → Mannose-6-P	Mannose Uptake
Pel Protein	manY	Mannose → Mannose-6-P	Mannose Uptake
Mannosephosphotransferase Enzyme IIB	manZ	Mannose → Mannose-6-P	Mannose Uptake
Xylose utilization			
Xylose Proton Symport Protein	xylE	Xylose transport	Xylose Uptake
Xylose Isomerase	xylA	Xylose → Xylulose	Xylose Catabolism
Xylulokinase	xylB	Xylulose → Xylulose-5-P	Xylose Catabolism

[0063] A cell that cannot utilize glucose as a carbon source can be created, for example, by disrupting the following three genes: *ptsG*; *manZ, manY* or *manX*; and *glk*. Such a strain will not be able to use mannose, either.

[0064] A cell that cannot use xylose as a carbon source can be created, for example, by disrupting one of the following genes: *xylA*, *xylB*, or xylE.

[0065] A cell that cannot utilize arabinose as a carbon source can be created, for example, by disrupting one of the following genes: *araA, araB, araF, araH,* or *araG.*

[0066] A cell that cannot use galactose as a carbon source can be created, for example, by disrupting one of the following genes: *galK, mglB, mglC,* or *mglA.*

[0067] A cell. that cannot use mannose as a carbon source can be created, for example, by disrupting one of the following genes: *manX, manY or manZ.*

[0068] Examples of substrate-selective strains useful in the method described herein include:

1. a) glucose-selective strain: mutations in *araA galK xylA manZ* (rendering the cell unable to utilize arabinose, galactose, xylose or mannose as a carbon source)
2. b) xylose-selective strain: mutations in *ptsG manZ glk araA galK* (rendering the cell unable to utilize glucose, mannose, arabinose or galactose as a carbon source)
3. c) arabinose-selective strain: mutations in *ptsG manZ glk xylA galK* (rendering the cell unable to utilize glucose, mannose, xylose or galactose as a carbon source)
4. d) galactose-selective strain: mutations in *ptsG manZ glk araA xylA* (rendering the cell unable to utilize glucose, mannose, arabinose or xylose as a carbon source)
5. e) mannose-selective strain: mutations in *araA galK xylA* (rendering the cell unable to utilize arabinose, galactose, or xylose as a carbon source; this cell can still utilize glucose as a carbon source as well as mannose).

Constructing yeast strains that are incapable of utilizing specific sugars

[0069] While many yeasts can utilize all of the sugars found in lignocellulosic hydrolysates, the notable exception is *Saccharomyces cerevisiae* which is unable to use pentose sugars as a carbon source. *S. cerevisiae* lacks several of the key enzymes involved in arabinose and xylose utilization. Yeast strains that are unable to utilize arabinose, galactose, or xylose, can be constructed by eliminating one gene from the respective sugar pathway as indicated in the following table. To construct a yeast strain that is incapable of utilizing glucose, either the HXT1, HXT2, HXT3, HXT4, HXT6, HXT7, and SNF3 genes or the GLK1, HXK1, and HXK2 genes must be eliminated.

Examples of pathways for sugar utilization in yeast

[0070]

Enzyme	**Gene**	**Reaction catalyzed**	**Pathway**
Arabinose utilization			
Aldose Reductase	GRE	Arabinose → Arabinitol	Arabinose Catabolism
Arabinitol-4-Dehydrogenase	LAD1	Arabinitol → Xylulose	Arabinose Catabolism
Xylulose Reductase	ALX1	Xylulose → Xylitol	Arabinose Catabolism
Xylitol Dehydrogenase	XYL2	Xylitol → Xylulose	Xylose Catabolism
Xylulokinase	XKS1	Xylulose → Xylulose-5-P	Xylose Catabolism
Galactose utilization			
Galactose Permease	GAL2	Galactose transport	Galactose Uptake
Galactokinase	GAL1	Galactose → Galactose-1-P	Galactose Catabolism
Galactose-1-P Uridyl Transferase	GAL7	Galactose-1-P → Glucose-1-P	Galactose Catabolism
UDP-Glucose-4-Epimerase	GAL 10	UDP-Glucose → UDP-Galactose	Galactose Catabolism
Glucose utilization			
Low-affinity glucose transporter	HXT1	Glucose transport	Glucose Uptake
High-affinity glucose transporter	HXT2	Glucose transport	Glucose Uptake
Low-affinity glucose transporter	HXT3	Glucose transport	Glucose Uptake

Glucose utilization			
High-affinity glucose transporter	HXT4	Glucose transport	Glucose Uptake
High -affinity glucose transporter	HXT6	Glucose transport	Glucose Uptake
High -affinity glucose transporter	HXT7	Glucose transport	Glucose Uptake
Plasma membrane glucose sensor	SNF3	Glucose transport	Glucose Uptake
Glucokinase	GLK1	Glucose → Glucose-6-P	Glucose Uptake
Hexokinase isoenzyme 1	HXK1	Glucose → Glucose-6-P	Glucose Uptake
Hexokinase isoenzyme 2	HXK2	Glucose → Glucose-6-P	Glucose Uptake
Xylose utilization			
Xylose Reductase	XYLI	Xylose → Xylitol	Xylose Catabolism
Xylitol Dehydrogenase	XYL2	Xylitol → Xylulose	Xylose Catabolism
Xylulokinase	XKS1	Xylulose → Xylulose-5-P	Xylose Catabolism

[0071] A yeast cell that cannot utilize glucose as a carbon source can be created, for example, by disrupting the following three genes: *GLK1, HXK1, and HXK2.* A yeast cell that cannot utilize glucose as a carbon source can also be created by disrupting the following seven genes: HXT1, HXT2, HXT3, HXT4, HXT6, HXT7, and SNF3.

[0072] A yeast cell that cannot utilize arabinose as a carbon source can be created, for example, by disrupting one of the following genes: *GRE, LAD1, or ALX1.* It is preferable not to delete the XYL2 or XKS1 genes since these genes are also utilized in xylose catabolism.

[0073] A yeast cell that cannot utilize galactose as a carbon source can be created, for example, by disrupting one of the following genes: *GAL2, GAL1, GAL7, or GAL10.*

[0074] A yeast cell that cannot utilize xylose as a carbon source can be created, for example, by disrupting one of the following genes: *XYL1, XYL2, or XKS1.*

Optimization of ethanol production

[0075] The substrate-selective cell used in the method described herein includes at least one metabolic pathway for the production of the desired product, for example ethanol. If the native, wild-type cell does not include an ethanol production pathway, genetic engineering can be used to introduce such a pathway so as to render the cell ethanologenic. If the native cell includes an ethanol pathway, the cell can optionally be further metabolically engineered to optimize the ethanol production pathway.

[0076] Optionally, the substrate-selective cell described herein is engineered to overexpress an alcohol dehydrogenase enzyme and a pyruvate decarboxylase enzyme. Pyruvate is converted by pyruvate decarboxylase to acetaldehyde, which is subsequently converted to ethanol by alcohol dehydrogenase. In some endogenous bacterial systems, the conversion of pyruvate to ethanol is rate-limiting in the production of ethanol, and results in excess substrate being converted to other byproducts such as acetic acid and succinic acid. The presence of high activities of alcohol dehydrogenase enzyme and pyruvate decarboxylase enzyme effectively increase the organism's ability to produce ethanol in order to maintain high conversion efficiency and minimize the amount of byproduct. One option for introducing these two enzymes into a substrate-selective cell is to transform the cell with a plasmid, such as pLOI308 (Ingram et al., 1987, Appl. Environ. Microbiol. 53:2420; Ingram and Conway, 1988, Appl. Environ. Microbiol. 54:397), that elevates ethanol formation in *E. coli.* This plasmid uses the *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase B genes. The cell may be transformed with a "production of ethanol" (PET) operon in which the alcohol dehydrogenase gene and the pyruvate decarboxylase gene from *Z. mobilis* have been placed under control of the Lac promoter. When expressed together, alcohol dehydrogenase gene and pyruvate decarboxylase cause ethanol to be the major product of *E. coli* fermentations.

[0077] Alternatively or additionally, ethanol production can be optimized by knocking out by-product pathways in order to guide additional substrates, and force more carbon, into the ethanol production pathway. By-product pathways genes that can be knocked-out in order to enhance ethanol production include, but are not limited to, pyruvate formate lyase, fumarate reductase, and lactate dehydrogenase.

Biological production of ethanol and other biochemicals

[0078] Biological conversion of organic material, such as a biomass, to a desired product, such as ethanol, can involve one or more of the following steps, in any order: harvesting the biomass, performing separation or extraction steps on the biomass, pretreating the biomass, hydrolyzing the biomass, contacting the biomass with substrate-selective cells described herein under conditions to convert a component of the hydrolysate into a desired product (i.e., fermentation), and subsequent isolation and/or purification of the product.

[0079] A biological approach to the production of ethanol typically involves four or five stages. A "pretreatment" phase can be used to make the lignocellulosic material such as wood or straw amenable to hydrolysis. Pretreatment assists in liberating the cellulose from the lignin and makes it more accessible to hydrolysis. Pretreatments are done through physical (mechanical) and/or chemical means. Pretreatment techniques include acid hydrolysis, steam explosion, ammonia fiber expansion, alkaline wet oxidation and ozone pretreatment.

[0080] Cellulose and hemicellulose molecules are long chains of sugar moieties. Hydrolysis is used to break down the long molecules in the cellulosic biomass into simple sugars, such as monomeric sugars, prior to subjecting the mixture to biological conversion of the sugars into the desired products. Hydrolysis can be effected using chemical, physical or enzymatic means. The most commonly used method of chemical hydrolysis is acid hydrolysis. Dilute acid can be used under high heat and high pressure, or more concentrated acid can be used at lower temperatures and pressures. The products from this hydrolysis are neutralized, and microbial fermentation as described herein can then be used to produce the desired product, such as ethanol. It should be noted that acid hydrolysis produces several potent inhibitors including acetic acid, furfural and hydroxymethyl furfural (HMF). Advantageously, the mixture can be cleared of inhibitors by contacting it with a cell that is selective for the inhibitor, as a carbon substrate, in accordance with the method described herein.

[0081] Alternatively or additionally, an enzymatic hydrolysis process can be used. Cellulose chains can be broken into glucose molecules by cellulase enzymes. Lignocellulosic biomass can be enzymatically hydrolyzed at a relatively mild condition (50 deg C and pH5), thus enabling effective cellulose breakdown without the formation of byproducts that would otherwise inhibit enzyme activity.

[0082] Optionally, prior to biological fermentation of the mixture described herein, the sugars in the hydrolysate are separated from residual materials, such as lignin. At this point the mixture is subjected to biological production of the product(s) using a plurality of substrate-selective cells as described herein. The resulting product, such as ethanol or lactate, may then be isolated and purified. In methods where ethanol is produced, isolation and purification can be accomplished by distillation.

[0083] Biological fermentation, which is sometimes termed "microbial fermentation" when microorganisms are used, yields the desired product, such as ethanol or lactate. It should be noted that the word "fermentation" as used herein is not limited to a strict biochemical meaning of an anaerobic process of converting carbon substrates into acids and alcohols; rather the word "fermentation" is used more broadly as in the art of industrial microbiology, such that it is meant to encompass any large-scale production of biochemicals using microorganisms. As such, a "fermentation" can be either aerobic or anaerobic. Contacting a lignocellulosic hydrolysate with the plurality of substrate-selective cells described herein under conditions to form a selected product is considered herein to be a "fermentation" regardless of whether the process is performed under aerobic or anaerobic conditions.

[0084] Biological fermentation, during which substrate-selective cells described herein are contacted with the biomass hydrolysate under conditions to convert a component of the hydrolysate into a desired product, may take any form, without limitation. For example, it may be a batch fermentation, a fed-batch fermentation, a steady-state fermentation or a continuous fermentation. Fermentation may be done under either aerobic or anaerobic culture conditions, or both in a sequential manner.

[0085] In case the method does not employ inhibitor-selective cells, biological processing of the hydrolyzed biomass may be carried out in a single bioreactor, in a co-fermentation that includes the plurality of sugar-selective cells and the hydrolyzed biomass. The plurality of sugar-selective cells operates independently and concurrently on the hydrolyzed biomass and, as noted elsewhere herein, can be responsive to changes in the relative proportions of the sugars in the fermenter. In examples that employ one or more inhibitor-selective cells in addition to sugar-selective cells, either a single stage process (all cells concurrently and independently operating on the biomass in a single bioreactor) or a dual stage process (inhibitor-selective cells operating on the biomass first, followed by contact with the sugar-selective cells) can be used, as set forth in more detail below.

[0086] The substrate-selective cells used in a particular stage of the fermentation process described herein may be either all bacterial cells, or all yeast cells. For example, in a dual stage process as described in more detail below, acetate-selective *E. coli* cells could be used in Stage 1 in a first reactor, followed by a second reactor in Stage 2 containing a collection of yeasts having various sugar-selectivities.

[0087] The efficacy of fermentation reactions is further complicated because hydrolysis of organic material continually generates compounds which inhibit the subsequent conversion of organic substrates such as sugars by fermentation. As discussed above, xylose is acetylated in lignocellulose (Timmel, 1967, Wood Sci. Technol. 1:45 ; Chesson et al., 1993, J. Sci. Food Agric. 34:1330), and therefore acetic acid, a particularly potent inhibitor of fermentation, is an unavoidable product of lignocellulose hydrolysis. Production of some inhibitors can be reduced by alterations in the hydrolysis process or in the organic material itself (e.g., through genetic engineering). However, it has to date not been feasible to eliminate all inhibitors. Not only must an efficient fermentation system be able to handle a mixture of substrates, but it must also be able to proceed in the fluctuating presence of some inhibitors such as acetic acid without loss of either yield or productivity. Previous approaches to convert mixtures of sugars and inhibitors have not been able to achieve the rate of conversion necessary to make the process economically viable.

[0088] Thus, in a method described herein that utilizes an inhibitor-selective cell (ISC), such as an acetate-selective cell (ASC), either a single stage or a dual stage (two step) fermentation can be employed.

[0089] In a single stage fermentation, the inhibitor-selective cell (e.g., an acetate-selective cell) and at least one, or at least two, sugar-selective cells are contacted with the hydrolysate concurrently.

[0090] In a dual stage fermentation (Fig. 1), the inhibitor-selective cell is first contacted with the hydrolysate in order to metabolize the acetic acid generated during hydrolysis (Stage 1). The hydrolysate is then contacted with the sugar-selective cells to metabolize the sugar monomers present in the mixture (Stage 2). Advantageously, the inhibitor-selective cells may lyse after the acetate is fully consumed, thereby providing additional nutrients for the sugar-selective cells.

[0091] In a process using acetate-selective cells in Stage 1, it is advantageous to maintain Stage 1 as a continuous (or fed-batch) process to maintain a zero acetate concentration as a result of substrate limitation. The process of this continuous process is then matched with Stage 2, which may be conducted batch fermentation, linear fed-batch fermentation, or exponential fed-batch fermentation (Example VIII). The two stage process may operate in fed-batch or continuous mode under carbon (i.e., acetate) limitation.

[0092] Acetate consumption in Stage 1 may be performed under aerobic conditions. Stage 2 can be anaerobic or aerobic, depending on the cells used and the identity of the biochemical being produced. Optimal production of the fermentation product ethanol or lactate, for example, during Stage 2 may be accomplished under anaerobic conditions. Thus, when the method described herein is used to produce ethanol or lactate, a dual stage fermentation may be when an optional inhibitor-selective cell is used.

[0093] Optionally, the cultures can be supplemented with additional nutrients in Stage 1, Stage 2, or both; or in the single stage when a single stage process is used.

[0094] The utilization of a two stage process that first eliminates the presence of inhibitors, then converts metabolic substrates into the desired fermentation product, increases both the rate of conversion and product yield while leaving little residual biomass hydrolysate unused.

[0095] Thus, as summarized above, and as illustrated and described in more detail in the following Examples, described herein is a method of concurrent conversion of hexose and pentose sugars which are present in lignocellulosic hydrolysates obtained from organic material. The problem is that a single microorganism is unable to metabolize multiple sugars concurrently. Instead, any given microorganism has a complex regulatory network which forces sugars to be metabolized sequentially. This sequential nature invariably reduces the overall rate of a fermentation process to generate the desired product.

[0096] Moreover, the fermentation of hexose and pentose sugars is further reduced by inhibitory compounds, such as acetate, which are unavoidable in the hydrolysate. Although the inability of microorganisms to utilize mixed sugars in the presence of acetate is most commonly associated with fuel ethanol production, the formation of other fermentation products (butanol, succinic acid, lactate, pyruvate, fumarate etc.) from biomass hydrolysates would similarly be greatly enhanced by a process that could eliminate acetate and effectively use a multitude of sugars.

[0097] Another significant disadvantage in current one-organism processes is that the metabolic pathways to convert glucose into a desired product at optimal yield and productivity do not generally correspond to the metabolic pathways to convert xylose into the same product. Ideally, a process converting xylose and glucose concurrently into a single product would permit these pathways to operate independently of one another, with glucose metabolism not influencing xylose metabolism and vice versa.

[0098] Thus addressed herein are many of the limitations that have plagued previous attempts to convert lignocellulosic biomass into ethanol and other desired products. The method described herein is able to simultaneously metabolize a variety of organic substrates, thereby eliminating the growth pattern demonstrated by a single microorganism in which it linearly consumes one substrate at a time. Additionally, while employing a number of metabolic alterations that, if made to a single organism, would result in an unstable cell that is prone to spontaneous mutations, these alterations are made in a plurality of different organisms, yielding a highly stable system. Furthermore, each cell within the plurality, by independently metabolizing a specific substrate, is able to fully utilize that substrate resulting in maximum yield of a single metabolic product, whereas a single cell attempting to metabolize a number of different substrates often yields a number of different products. By concurrently metabolizing multiple organic substrates, inhibitors are depleted, production efficiency is improved, and yields of fermentation products are enhanced. The process described herein is able to handle varying mixtures of sugars and is also able to contend with the fluctuating presence of some inhibitors such as acetic acid without loss of yield or productivity.

[0099] The disclosure provides a plurality of cells capable of both eliminating inhibitors and concurrently converting hexose and pentose sugars into a desired fermentation product. Described herein are methods of making and using the various metabolically engineered cellsdescribed herein.

[0100] The present invention is illustrated by the following examples.

EXAMPLES

Example I.

Co-fermentation strategy to consume sugar mixtures effectively

Introduction

[0101] We report a new approach for the simultaneous conversion of xylose and glucose sugar mixtures into products by fermentation (Eiteman et al., 2008, J. Biol. Engineering, 2:3). The process simultaneously uses two substrate-selective strains, one which is unable to consume glucose and one which is unable to consume xylose. The xylose-selective (glucose deficient) strain CGSC5457 (ZSC113) has mutations in the *glk, ptsG* and *manZ* genes while the glucose-selective (xylose deficient) strain ALS1008 has a mutation in the *xylA* gene. By combining these two strains in a single process, xylose and glucose are consumed more quickly than by a single-organism approach. Moreover, we demonstrate that the process is able to adapt to changing concentrations of these two sugars, and therefore holds promise for the conversion of variable sugar feed streams, such as lignocellulosic hydrolysates.

[0102] The goal of this study was to characterize xylose-selective and glucose-selective strains of *Escherichia coli.* Specifically, we set out to construct *E. coli* strains which independently will only consume xylose or glucose without any loss in consumption rate and which can be used together, acting in concert to consume sugar mixtures effectively.

Materials and Methods

Strains

[0103] *Escherichia coli* strains MG1655 (wild-type, F-, λ), CGSC5457 (ZSC113, *lacZ827*(UGA) or *lacZ82*(Am) *ptsG22 man*Z12 *glk-7 relA*1 *rpsL223*(strR) *rha*-4), DY330 (Δ*lac*U169 *gal*490 λc1857 Δ(*cro-bioA*)), and ALS1008 (MG1655 *xylA*::*Tet*) were used in this study. CGSC5457 (ZSC113) was obtained from the *E. coli* Genetic Stock Culture (Yale University). DY330 contains a lambda cl857 lysogen and is used to induce the genes which constitute the lambda Red recombination system (Yu et al., 2000, Proc Natl Acad Sci USA. 97:5978).

Generating the xyla::Tet knockout

[0104] The *xylA* gene which encodes D-xylose isomerase was knocked out using the lambda Red recombination system. Primers were designed which could amplify the *tetA* gene and promoter from pWM41 (Metcalf et al., 1996, Plasmid. 35:1.) bracketed by the first and last 50 bases of the *xylA* coding sequence. The *tetA* gene codes for the tetracycline resistance protein. The forward primer 5' ATGCAAGCCTATTTTGACCAGCTCGATCGCGTTCGTTATGAAGGCTC AAAACATCTCAATGGCTAAGGCG 3' (SEQ ID NO:1) contains the first 50 bases of the *xylA* coding sequence followed by bases 1349 - 1368 of TRN10TETR (Accession Number J01830) from pWM41 while the reverse primer 5' TTATTTGTCGAACAGATAATGGTTTACCAGATTTTCCAGTTGTTCCTG GCGGC TGGTTTATGCATATCGC (SEQ ID NO:2) 3' contains the last 50 bases of the *xylA* coding sequence followed by bases 3020 - 3039 of TRN10TETR from pWM41. The bases from pWM41 are underlined in the primers. The two primers were used to amplify a 1,791 bp fragment from pWM41 DNA using the polymerase chain reaction (PCR) with *Pfu* polymerase. The resulting DNA was gel-isolated and electroporated into DY330 electrocompetent cells which were prepared as described (Yu et al., 2000, Proc. Natl. Acad. Sci. USA 97:5978). Tet(R) colonies were then selected. The presence of the *xylA::Tet* knockout was confirmed by the inability of DY330 *xylA::Tet* to grow in minimal xylose media. ALS1008 (MG1655 *xylA*::*Tet)* was constructed by transducing *xylA*::Tet from DY330 *xylA*::Tet into MG1655 using P1 transduction.

Growth conditions

[0105] For each bioreactor experiment, a single strain was first grown in a tube containing 10 mL BXG medium, then 5 mL transferred to 50 mL BXG medium in a 250 mL shake flask. All flasks were incubated at 37°C and 250 rpm (19 mm pitch). For those fermentations in which a single strain was used, when the OD of the shake flask culture reached approximately 4, the contents of the shake flask were diluted with BXG medium so that 100 mL having an effective OD of 2.0 was used to inoculate the fermenter. For those experiments in which two strains were used in a single fermentation, the contents of two shake flasks were diluted with BXG medium to 100 mL so that each strain had an effective OD of 2.0 (i.e., in the 100 mL volume). Basal medium contained (per L): 13.3 g KH_2PO_4, 4.0 g $(NHa)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$,13.0 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1.5 mg $CuCl_2 \cdot 2H_2O$, 15.0 mg $MnCl_2 \cdot 4H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 100 mg Fe(III)citrate, 8.4 mg $Na_2EDTA \cdot 2H_2O$, 1.7 g citric acid, and 0.0045 g thiamine-HCl. BXG medium comprised basal medium with 15 g/L glucose and 8 g/L xylose. Shake flask media were adjusted to a pH or 7.0 with 20% NaOH.

Fermentation

[0106] Batch experiments were carried out in a 2.5 L bioreactor (Bioflow 2000, New Brunswick Scientific Co. Edison, NJ, USA) containing 1.0 L BXG medium. Throughout aerobic fermentations, air was sparged into the fermenter at a flowrate of 1.0 L/min, and the agitation was 1000 rpm to ensure no oxygen limitation. In some experiments an anaerobic phase was initiated after aerobic growth. For these cases additional xylose and/or glucose was supplied as reported. To maintain anaerobic conditions, carbon dioxide was provided at a flowrate of 0.2 L/min, and the agitation was 150 rpm.

[0107] Fed-batch experiments were carried out in the same vessel initially containing 1.0 L basal medium (no xylose and glucose). Immediately after inoculation, a feed containing a mixture of xylose and glucose without additional medium components commenced as reported. This medium was fed at an exponentially increasing rate designed to achieve a growth rate of 0.1 h^{-1} for a substrate concentration of 30 g/L.

[0108] For all fermentations, the pH was controlled at 6.7 using 15% (w/v) NH_4OH, and the temperature was controlled at 37°C.

Analyses

[0109] The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth, and this value was correlated to dry cell mass. Previously described liquid chromatography methods were used to quantify xylose and glucose (Eiteman et al., 1997, Anal. Chem. Acta 338:69) and other organic compounds (Chesson et al., 1993, J. Sci. Food Agric. 34:1330).

[0110] The fraction of the microbial population that constituted each strain was determined by plating serial dilutions of cultures onto both LB and LB-tetracycline plates.

Results

Aerobic utilization of xyloselglucose mixtures

[0111] *Escherichia coli* ZSC113 and *E. coli* ALS1008 are unable to consume glucose and xylose, respectively. The xylose-selective strain ZSC113 has mutations in the three genes involved in glucose uptake (Curtis and Epstein, 1975 J Bacteriol. 122:1189), rendering it unable to consume glucose: *ptsG* codes for the Enzyme $IICB^{Glc}$ of the phosphotransferase system (PTS) for carbohydrate transport (Postma et al., 1993 Microbiol Rev. 57:543), *manZ* codes for the IID^{Man} domain of the mannose PTS permease (Huber and Erni, 1996, Eur J Biochem. 239:810), *glk* codes for glucokinase (Curtis and Epstein, 1975 J Bacteriol. 122:1189). We constructed strain ALS1008 which has a knockout in the *xylA* gene encoding for xylose isomerase, rendering ALS1008 unable to consume xylose. In a medium composed of a mixture of these two sugars, ZSC113 would be expected to consume the xylose selectively while ALS1008 should exclusively consume the glucose. We first sought to verify these expectations in three aerobic batch experiments.

[0112] In a first (control) experiment, a defined medium containing both 8 g/L xylose and 15 g/L glucose was inoculated with a single wild-type strain, MG1655, and grown aerobically (Fig. 2). The glucose/xylose mixture was chosen to reflect the concentrations of glucose and xylose that are found in typical lignocellulosic hydrolysates. As expected, we observed diauxic growth as reported by many other studies when a single strain is inoculated into a medium containing two or more carbon sources. The important observations were that glucose and xylose were consumed sequentially, and that the complete consumption of this mixture required about 8.5 hours.

[0113] In a second set of aerobic experiments, the same defined medium containing two carbon sources was inoculated with one or the other of the two strains, ZSC113 or ALS1008. In the fermenter inoculated with only ZSC113 (Fig. 3a), 8 g/L xylose was completely consumed in 7 h and the OD reached 10, In this case, the concentration of glucose remained unchanged. In the fermenter containing only ALS1008 (Fig. 3b), we observed the complete consumption of 15 g/L glucose in 7.5 h with the OD reaching 15, while the concentration of xylose remained unchanged. As expected the two strains each consumed only one of the sugars, leaving the other carbohydrate unconsumed.

[0114] In a third aerobic batch experiment, we inoculated both ZSC 113 and ALS1008 into a single fermenter containing 8 g/L xylose and 15 g/L glucose. For this co-culture fermentation, glucose was consumed in 7.5 h, and xylose was simultaneously consumed in 7.0 h (Fig. 4). Moreover, the final OD of this mixed culture was about 25, identical to the sum of the ODs achieved in the fermentations in which one or the other carbohydrate was used. Thus, each strain appears to grow and consume its substrate independently. The combined process (i.e., consuming both sugars simultaneously) occurred at the same rate as the two individual processes so that each consumption rate was unaffected by the presence of the other carbohydrate. Compared to the wild-type (single organism) process, this process required about 15% less time to consume the same carbohydrate mixture aerobically, and moreover each substrate was consumed independently. The single-organism process (Fig. 2) was completely different than the dual-organism process (Fig. 4) in which both carbon sources were consumed simultaneously. No products were observed in these batch fermentations.

Aerobic fed-batch utilization of xyloselglucose mixtures

[0115] When a microorganism grows in a substrate-limited fashion (for example, in a fed-batch process), the growth rate is controlled by the rate that the limiting substrate is supplied. Moreover, the concentration of that substrate remains at zero. In a bioprocess with two substrate-selective organisms which are both under carbon-limiting conditions, each organism should independently be controlled by and adapt to the quantity of the carbon source present that it can consume. We wished to test this hypothesis using a fed-batch process in which the two-carbohydrate feed increased exponentially at a nominal rate of 0.1 h^{-1}, far below the maximum growth rate of either strain. Moreover, in addition to the flowrate exponentially increasing to maintain a fixed specific growth rate, the composition of the feed changed in discrete shifts in order to simulate a variable concentration that might be encountered in a real process. Specifically, for the first 20 h we maintained feed concentrations at 20 g xylose/L and 30 g glucose/L (20:30). At 20 h, this feed was replaced by feed concentrations of 30:30, at 30 h to 30:60, and then finally to 20:60 at 40 h. At 20 h, 30 h, 40 h and 50 h we determined the fraction of the population which was the glucose-consuming strain ALS1008 (and thus by difference the fraction which was the xylose-consuming strain ZSC113).

[0116] During the entire fed-batch process, the xylose and glucose concentrations in the fermenter remained at zero (Fig. 5), demonstrating that each substrate individually limited the process. Moreover, the distribution of the microbial population responded in unison with the shift in substrate concentrations. At 20 h, after the process had acclimated to a 20:30 xylose:glucose composition (g/L), the population was 35% ZSC113 (i.e., the xylose-consuming strain). Ten hours after the feed composition shifted to 30:30, the population was 50% ZSC113. Similarly, ten hours after the feed composition shifted to 30:60, the population returned to 42% ZSC 113, and then ten hours after the feed had become 20:60, the population decreased to 32% ZSC 113. These results demonstrate that the process adjusts the distribution of strains to match the distribution of substrates.

Anaerobic product formation with xylose/glucose mixtures

[0117] Wild-type *E. coli* is a mixed acid fermenter, and generates acetate, lactate, formate, ethanol and succinate under anaerobic conditions, with the yield of each depending on the strain and carbon source (Clark, 1989, FEMS Microbiol. Rev. 63:223). The strains used for this study of substrate-selective uptake did not generate an elevated concentration of any product during the aerobic studies (beyond the expectation for wild-type strains). ZSC113 and ALS1008 do not have any additional mutations which would cause the product distribution to be different from the wild-type parent MG1655. We thus wanted to examine how ZSC113 and ALS1008 behaved under anaerobic conditions in which products would accumulate. Three experiments were conducted under anaerobic conditions, analogous to those conducted under aerobic conditions previously.

[0118] In a first experiment, wild-type MG1655 was grown under aerobic conditions as before to consume 15 g/L glucose and 8 g/L xylose. When both substrates were nearly consumed (after about 8.5 h as shown in Fig. 2), we added enough glucose and xylose into the fermenter approximately to return both concentrations to their initial levels. Anaerobic conditions were initiated under an atmosphere of 100% CO_2, and the products were measured during the anaerobic growth phase (Fig. 6). In this culture of a single strain (having an OD of 21) 10 g/L of glucose was consumed in about 2.5 h (4 g/Lh), equivalent to a specific glucose consumption rate of 630 mg/gh. Initially xylose was consumed at a rate of 310 mg/gh (2 g/Lh). However, after 4 h of anaerobic conditions, the xylose consumption rate decreased to less than 150 mg/gh (1 g/Lh), and continued to slow. Nearly 4 g xylose/L remained after 9 h of anaerobic conditions. It must be noted that the organism consumed glucose then xylose in the aerobic phase preceding these anaerobic conditions (as shown in Fig. 2), and therefore at the time of the switch to anaerobic conditions the xylose-consuming pathways were fully induced. One explanation for the substantial decrease in xylose consumption rate is the sensitivity of xylose-degradation to the presence of acetate as previously reported for yeast (Helle et al., 2003, Enzyme Microbial Technol. 33(6):786; van Zyl et al., 1991, Enzyme Micro. Technol. 13:82). This explanation is supported by the observation that xylose consumption continued to slow even after glucose was depleted under anaerobic conditions.

[0119] In a second experiment, the two substrate-selective strains ALS1008 and ZSC113 were grown individually on the mixed substrate medium, the one depleted substrate added back, and then anaerobic conditions commenced. For the case of the xylose-consuming strain ZSC113, xylose was consumed at a constant rate of 1.4 g/Lh during the anaerobic phase and glucose was not consumed (Fig. 7a). This single organism was present only at an OD of 9.5, so that on a specific basis the xylose consumption rate was 500 mg/gh, greater than the highest rate observed in the xylose portion of the fermentation using the wild-type MG1655. For the experiment in which the glucose-consuming strain ALS1008 was inoculated into the mixed substrate medium, glucose was exclusively consumed during the anaerobic phase at a constant rate of 3 g/Lh, and xylose was not consumed (Fig. 7b). In this case, the specific glucose consumption rate was about 770 mg/gh, greater than the rate we observed for the wild-type MG1655 during the anaerobic phase (i.e., Fig. 6). These two separate fermentations demonstrate that the strains will each consume only one substrate under anaerobic conditions, and that they will consume this substrate slightly faster on a specific basis than the wild-type strain would under identical conditions.

[0120] In a third experiment we first simultaneously grew both strains in the mixed substrate medium under aerobic conditions. At the end of the 7.0 h aerobic growth phase, we added enough of both carbohydrates to return them to their initial concentrations, and anaerobic conditions were initiated. In this process, both xylose and glucose were quickly consumed (Fig. 8). Although we did not measure the proportion of the two strains, from previous aerobic results using one substrate (i.e., Fig. 3), we estimate that the OD of ZSC113 was about 13 and the OD of ALS1008 was about 9. Over the first two hours of the anaerobic phase, the xylose consumption rate was therefore about 475 mg/gh, while the glucose consumption rate was about 1300 mg/gh. The key point in these results is that the two-strain process is much faster than an otherwise identical single-strain process.

[0121] The products formed from the two-sugar fermentation were the same as those generated during either one of the single-sugar fermentations, although the distribution of products changed slightly. For example, the succinate yield from xylose using ZSC113 was 0.48 mol/mol, while the succinate yield from glucose using ALS1008 was 0.30 mol/mol. From the sugar mixture, the observed succinate yield by two organisms was 0.41 mol/mol sugar consumed, a value between the yields of the individual strains on the two substrates. For all three cases, formate was generated with the highest yield (1.24 mol/mol glucose, 1.58 mol/mol xylose, and 1.46 mol/mol sugar mixture), and lactate was generated the least.

[0122] One limitation of this study was that high concentrations of acid products are known to inhibit growth and substrate consumption rates. This phenomenon would tend to affect the mixed culture more than either single-sugar culture, since for the former case both sugars would quickly be converted into more mixed acid products. This substrate-selective approach may perform significantly better for strains specifically designed to accumulate a single product such as ethanol which does not cause acid inhibition.

Discussion

[0123] The process described in this study offers a new approach for the simultaneous conversion of sugar mixtures into microbial products such as ethanol. The key characteristic of this approach is the use of multiple strains which are each selective in their consumption of a carbon source. *Excluding* substrate consumption in a strain by gene deletions represents an innovative shift from the long-studied approach of constructing a "do-it-all" organism for the conversion of multiple substrates into a desired product.

[0124] There are two significant advantages that the process has for the simultaneous conversion of sugar mixtures, as exemplified by xylose and glucose. Most importantly, as demonstrated by the fed-batch process (Fig. 5), the system adapts to fluctuations in the feed stream, i.e. cultures actually grow in concert with the feed composition. Since they are each specialists, the strains can not adversely compete with each other for the consumption of the substrates. Using a fed-batch process prevents sugar accumulation, and permits each strain to convert its target sugar at high yield and productivity. Operational robustness is the hallmark of this process strategy, and it constitutes a major advance toward the utilization of lignocellulosic biomass. Second, although not part of this study, additional metabolic engineering strategies can focus on improving the individual production strains independently. For example, work can now be devoted to improving the glucose-selective strain for ethanol production with minimal concern for how these changes would impact the conversion of xylose. We do not need to compromise one objective for another.

[0125] Gene knockouts affecting only one carbohydrate consumption pathway appeared not to have deleteriously impacted the consumption of the other carbohydrate. Indeed, previous results have demonstrated improved xylose utilization in sugar mixtures by the *ptsG* knockout alone (Kimata et al., 1997, Proc Natl Acad Sci USA. 94:12914; Nichols et al., 2001, Appl Microbiol Biotechnol. 56:120). In this study, catabolite repression due to the presence of glucose was made irrelevant by the use of two strains, since one cannot utilize glucose at all.

[0126] The results demonstrate that a population of substrate-selective strains, in which each individual strain only consumes a single sugar, is better able to metabolize a sugar mixture than a single strain consuming multiple sugars. Other than the mutations involving substrate consumption, the strains used for this study did not contain additional mutations which would cause them to generate a product preferentially. The next step would be to use this approach with microbial strains specifically modified to accumulate a desired product such as ethanol. This approach could potentially be extended to construct additional strains capable of the exclusive consumption of other sugars (e.g., arabinose) or inhibitors such as acetic acid and furfurals that are frequently found in lignocellulosic hydrolysates.

Example II.

Consumption of acetate by *Escherichia coli*

[0127] *E. coli* readily consumes acetic acid as a sole carbon/energy source (El-Mansi et al., 2006, Curr. Opin. Microbiol. 9:173), but it generally will not consume acetate in the presence of other substrates from which the cells can derive more energy. Of course, the presence of acetate diminishes the rates at which other substrates are consumed. However, *E. coli* can be forced to grow on acetate and prevented from consuming glucose or xylose (for example) by knocking out the genes which encode for glucose *and* xylose consumption. We refer to such a strain as "acetate-selective" because of the three substrates, acetate is its exclusive carbon nutrient. When exposed to a hydrolysate containing acetate, xylose and glucose, only the acetate will be consumed. This approach is merely an extension of the substrate-selective concept introduced above for xylose and glucose mixtures.

[0128] We tested whether acetate could be selectively removed from a mixture of xylose, glucose and acetate. We used *E. coli* MG1655 to generate ALS1060. MG1655 is a common wild-type strain (Jensen, 1993, J. Bacteriol. 175:3401), and we verified that it grows aerobically with acetate as the sole carbon source at a growth rate of approximately 0.24 h^{-1}. ALS1060 has four knockouts of genes coding for proteins involved in xylose and glucose utilization (these genes were described above): *ptsG, manZ, glk,* and *xylA.* These four mutations should prevent the consumption of either xylose or glucose by ALS1060, but have no known affect on acetate metabolism.

[0129] We grew ALS1060 in a batch process using a medium containing an extremely high proportion of acetate: 10 g/L acetate, 10 g/L xylose and 20 g/L glucose. In this case, ALS1060 consumed 10 g/L acetate at a growth rate of 0.16 h^{-1}, but left xylose and glucose unconsumed even after 40 hours (Fig. 9). The significant lag phase observed in this experiment can be attributed to using the poor MG1655 strain and the sudden exposure to 10 g/L acetate. Acetate consumption depends on the cellular balance between the glyoxylate shunt and isocitrate dehydrogenase (Holms, 1986, Curr Top Cell Regul 28:69), and different *E. coli* strains would be expected to behave quite differently. Our selection of MG1655 was merely to provide evidence that the concept would work; this strain turned out not to be a good acetate-grower. We have recently found *E. coli* strains which consume acetate at a growth rate over 0.70 h^{-1} with no lag, and will use these strains to develop the acetate-selective strain as described in the experimental approach.

[0130] In the preliminary experiments reported in Examples I and II we have been able to 1) remove acetate selectively from a mixture of xylose, glucose and acetate and 2) consume xylose and glucose simultaneously in a mixture of these two sugars. These steps can be linked together in a two-stage process to generate a product like ethanol, as conceptualized in Fig. 1 for an acetate/xylose/glucose mixture. After removal of acetate in Stage 1, the remaining mixture is subsequently fermented in a second process stage (Stage 2) to the desired product. Because knock-out strains are very stable, the strategy can readily be extended to any number of substrates. For example, an arabinose-selective strain will be unable to consume xylose and glucose, etc.

Example III.

Two stage fermentation

[0131] Rather than try to develop a single organism to accomplish all the process design goals required for lignocellulosic conversion, our novel approach uses multiple strains to do tasks efficiently and independently. Note that no competition exists in the envisioned co-culture (in Stage 2, Fig. 1). Competition involves multiple species competing for the same substrate. In this case, the strains each seek only their specific substrate and, being otherwise the same, do not interfere with each other. Thus, potential shortcoming from competition in a "mixed-culture" bioprocess is avoided.

[0132] There are several significant advantages that the envisioned continuous/fed-batch process has for the elimination of acetic acid and the simultaneous conversion of sugar mixtures, as exemplified by xylose and glucose. First, because the acetate-selective strain cannot grow in the absence of acetic acid, these cells will ultimately lyse in the second stage. By lysing, the cellular nutrients derived from acetate utilization are available to support growth of cells present in Stage 2. So, the inhibitor actually enhances product formation by enriching the hydrolysate with growth nutrients. It does not matter if the cells consume additional nutrients such as nitrogen, phosphorus, sulfur. These nutrients are conserved and become readily available to the organisms in the second stage. The goal of the first stage is only to consume acetate (quickly) and not consume any sugars.

[0133] Secondly, metabolic engineering strategies can focus on improving the individual production strains independently. For example, additional work can be devoted to improving the glucose-selective strain for ethanol production without concern for the impact of these changes on the conversion of xylose or on acetate tolerance/degradation. We do not need to compromise one objective for another.

[0134] Finally, the system adapts to fluctuations in the feed stream; that is, cultures actually grow in concert with the feed composition. For example, the system would respond to an increase in acetate concentration merely by increasing the cell density in Stage 1. Regardless of the perturbation of acetate in the feed (within a large range, as long as the cells remain carbon limited), this inhibitor will be completely removed in Stage 1. Similarly, as we have already observed, the system responds to an increase in the feed xylose concentration by increasing the cell density of the xylose-selective strain in Stage 2 (with no change in the cell density of any other strain). Using a fed-batch process would prevent sugar accumulation, and each strain would convert its target sugar at high yield and productivity. Operational robustness is the hallmark of this process strategy, and it constitutes a major advance toward the utilization of lignocellulosic biomass.

Example IV.

Concurrent Use of Sugars by *Escherichia coli*

[0135] In Example I we demonstrated that the concept of substrate-selective uptake works. Using *E. coli* we demonstrated that a xylose-selective strain will consume only xylose and a glucose-selective strain consumes only glucose in a mixture of these two sugars and without impacting each other. Moreover, in Example II we demonstrated that acetate can be selectively removed from a mixture of acetate, glucose and xylose.

[0136] The strains used in these studies were not isogenic, not derived from optimally-growing strains and did not generate any product such as ethanol. We will construct a series of substrate-selective strains and demonstrate the two-step process shown in Fig. 1 on simulated and actual hydrolysates. Strains are constructed based on gene deletion and/or heterologous gene transformation, then their behavior in controlled bioreactors is examines. After initial examination in bioreactors, additional molecular biology may be necessary to further improve or optimize the strain.

[0137] The following examples describe various aspects of the present disclosure:

Example IV. Concurrent use of sugars by *Escherichia coli*

Example V. Introduction of ethanol pathways into the strains

Example VI. Fermentation of simulated sugar mixture

Example VII. Consumption of acetate by *Escherichia coli* without sugar degradation

Example VIII. Simulated and real hydrolysates

Example IX. Concurrent use of xylose and glucose by Lactic Acid Bacteria

[0138] Like many sugars, glucose must first be phosphorylated before it can be further metabolized by *E. coli.* The principal route to phosphorylate glucose is by the phosphotransferase system (PTS). Because the PTS phosphorylates several sugars, some of the enzymes involved have broad specificities and can phosphorylate more than one sugar (Postma et al., 1993, Microbiol Rev. 57:543). Glucose can be phosphorylated by two different enzymes of the PTS, glucosephosphotransferase and mannosephosphotransferase encoded respectively by the *ptsG* and *manZ* genes (originally designated as *gpt* and *mpt*). Furthermore, glucose can be phosphorylated by the enzyme glucokinase which is encoded by the *glk* gene (Curtis and Epstein, 1975, J. Bacteriol. 122(3):1189). Strains that are unable to use glucose as a carbon source can be constructed by knocking out the genes encoding for glucosephosphotransferase (*ptsG*), mannosephosphotransferase (*manZ*) and glucokinase (*glk*).

[0139] Xylose is transported into *E. coli* by the xylose transport system and once internalized must be isomerized to xylulose by xylose isomerase and phosphorylated to xylulose-phosphate by xylose kinase before it can be metabolized (David and Weismeyer, 1970, Biochim. Biophys. Acta. 201:497). Strains that are unable to utilize xylose as a carbon source can be constructed by knocking out the gene encoding for xylose isomerase (*xylA*).

[0140] Arabinose is another sugar found in biomass hydrolysates, accounting for about 5% of the total sugar. The consumption of arabinose into *E. coli* is essentially analogous to the xylose uptake process. Arabinose consumption can be eliminated by knocking out the *araA* gene.

[0141] The hexose galactose is a component in lignocellulosic hydrolysate, but is commonly found at concentrations of 1% or less (Lee, 1997, Appl. Microbiol. Biotechnol. 65:56). Galactose uptake is mediated by galactokinase encoded by the *galK* gene, and galactose uptake is eliminated in a *galK* mutant. Because the composition of galactose in hydrolysate is so low, eliminating galactose uptake may be unnecessary.

[0142] The presence of the hexose mannose in lignocellulosic hydrolysate varies widely between 0-12% depending on the biomass (Lee, 1997, Appl. Microbiol. Biotechnol. 65:56). Mannose is phosphorylated exclusively by the mannose-specific components of the PTS (e.g., mannosephosphotransferase) encoded by *manX, manY* and *manZ*. Preventing glucose uptake by the triple mutations as described above (*manZ*) also prevents mannose consumption. Similarly, knocking out *xylA* and *araA* (and *galK*) has no impact on mannose consumption. Using the strategy outlined so far, we cannot construct a strain which consumes mannose but not glucose.

[0143] In order to consume all the sugars present in lignocellulosic hydrolysates simultaneously, strains are constructed which are expected to consume each one of these sugars alone. Thus, the following strains will be constructed:

1. a) glucose-selective strain: mutations in *araA galK xylA manZ*
2. b) xylose-selective strain: mutations in *ptsG manZ glk araA galK*
3. c) arabinose-selective strain: mutations in *ptsG manZ glk xylA galK*
4. d) galactose-selective strain: mutations in *ptsG manZ glk araA xylA*
5. e) mannose-selective strain: mutations in *araA galK xylA* or *araA galK xylA ptsG glk* (these strains will also consume glucose)

[0144] Our preliminary experiments (Examples I and II) were conducted in *E. coli* K12 or its non-isogenic derivatives, and included xylose and glucose, not the two other uniquely consumed sugars galactose and arabinose. The construction of a completely substrate-selective strain requires knocking out all other uptake systems. So, for example, to construct a xylose-selective strain, we knock out the *glk*, *ptsG*, *manZ*, *galK* and *araA* genes in the *E. coli* B strain. The B strain has been chosen for this project because it is a widely used prototrophic derivative of wild-type *E. coli* that is well characterized and grows rapidly in both defined and complex media. Furthermore, our experience is that this strain grows very well on a variety of carbon sources (e.g., xylose, arabinose, etc.). Other research groups have also favored derivatives of the B strain (Ingram et al., 1999, Biotechnol. Prog. 15:855; Tao et al., 2001, J. Bacteriol. 183(10):2979). The *E. coli* gene knockouts are constructed using the Keio collection of single-gene knockout mutants (Baba et al., 2006, Mol. Syst. Biol. 2:2006.0008). The lambda Red recombination system (Yu et al., 2000, Proc Natl Acad Sci U S A 97:5978 ; Datsenko and Wanner, 2000, Proc Natl Acad Sci U S A. 97:6640) may also be employed to construct the required gene knockouts. For control purposes substrate-selective strains that we construct can be compared to similar publicly available strains (such as CGSC5457 from the *E. coli* Genetic Stock Center that contains mutations in the *glk, ptsG,* and *manZ* genes and thus also can not consume glucose). The 4 substrate-selective B strains (one for each of glucose, xylose, arabinose and galactose) are expected to be useful platforms for other researchers to develop processes for the formation of various products from the five sugars (including mannose).

[0145] Additionally, using the same approach we will construct 4 substrate-selective using the ethanologenic *E. coli* strain "KO11." The result is expected to be eight substrate-selective strains (four each derived from B and KO11).

Example V.

Introduction of ethanol production pathways into sugar-selective *E. coli* cells

[0146] Decades of research have led to great improvements in strains of *E. coli* that accumulate ethanol, and the approach described herein can be adapted to current or future *E. coli* ethanologenic strains. The approach can be adapted readily to strains of *E. coli* which generate any other compound.

[0147] The sugar-selective KO11 strain of Example IV is expected to be ethanologenic, since KO11 is ethanologenic. To make the sugar-selective B strain of Example IV ethanologenic, ethanol pathways will be introduced into those strains. The four sugar-selective B strains will be transformed with pLOI308 (Ingram et al., 1987, Appl. Environ. Microbiol. 53:2420; Ingram and Conway, 1988, Appl. Environ. Microbiol. 54:397), one of the better characterized plasmids that elevates ethanol formation in *E. coli.* This plasmid uses the *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase genes. In *Zymomonas mobilis,* pyruvate is converted by pyruvate decarboxylase to acetaldehyde, which is subsequently converted to ethanol by alcohol dehydrogenase. Together these enzymes comprise nearly 10% of the soluble protein of wild-type *Z. mobilis* cells grown with glucose as the substrate (Doelle et al., 1993, Crit. Rev. Biotechnol. 13:57), enabling this organism to maintain a high conversion efficiency to ethanol.

[0148] The result is expected to be two sets of ethanologenic, sugar-selective strains of *E. coli* (four each derived from B and KO11; 8 total).

Example VI.

Fermentation of simulated sugar mixtures.

[0149] This example relates to the characterization of processes to metabolize sugar mixtures, ultimately in the presence of acetic acid (as described below in a subsequent section). Initial studies will be performed on synthetic mixtures of 2-5 sugars (selected from the list of mannose, galactose, glucose, xylose and arabinose), while subsequent studies will be conducted on real hydrolysates supplemented with additional nutrients as required (e.g., nitrogen and phosphate sources). These studies will use the 8 strains generated from Example V, with 4 strains present in a co-culture at the same time when all 5 sugars are present.

[0150] We will first establish the operating ranges for the processes through a series of chemostat experiments. While not generally used in industry, chemostat experiments provide an extremely useful way of determining the parameters necessary to design a relevant process (for example, a fed-batch process which is commonly used.) Like all fermentation experiments, these studies will involve highly-instrumented bioreactors in which feed-rate, temperature, pH, nutrient levels, oxygenation, etc. can be controlled. By studying a range of controlled growth rates, we will determine for each strain the biomass yields, specific rates of consumption/production of dissolved and gaseous compounds, and the maintenance energy requirements resulting from the various genetic perturbations. Like any cells growing on different carbon-sources, these strains will have differing maximum growth rates. Note that in a chemostat the microbial growth rate is determined by the nutrient feed rate (dilution rate), but the biomass concentration is determined by the limiting nutrient concentration. These maximum growth rates will help establish the maximum feed-rate of an envisioned fed-batch process. Operating a biological process at the maximum growth rate does not in general result in the maximum practical product formation rate for a variety of reasons such as oxygen requirement (for an aerobic process), heat duty, reduced biomass yield, genetic regulation of cells, etc. We will study the strains individually on single-substrate media, then single strains on dual-substrate media, then multiple strains on dual-substrate media. Drug resistances introduced into some strains will serve as selective markers to permit us to quantify the fraction of each strain comprising the total population.

[0151] In addition to the principal carbohydrates, major and minor fermentation products including ethanol, acetate, lactate, formate, succinate will be analyzed. We anticipate that significant lactate and formate will be generated for the B-derived ethanologenic strains, and in this case they may be further modified by deleting the enzymes responsible for these products: deleting the *ldh* gene which encodes lactate dehydrogenase will eliminate lactate from being produced while deleting the *pfl* gene which encodes pyruvate formate lyase will eliminate formate. We have used this strategy to increase the fermentation yields of several products in *E. coli* (Tomar et al., 2003, Appl. Microbiol. Biotechnol. 62:76; Lee et al., 2004, Appl. Microbiol. Biotechnol. 65:56; Smith et al., 2006, Appl. Microbiol. Biotechnol. 28:1695; Zhu et al., 2007, Appl. Environ. Microbiol. 73:456). The chemostat experiments will provide us with parameters to enable the study and implementation of a fed-batch process. We will feed in the mixed-sugar stream in a carbon-limited fashion (that is, each one of the four strains will be carbon limited for their sugar substrate). During an initial process "phase" cells will grow aerobically, while in a subsequent production phase, reduced oxygen availability will direct most of the carbon to the product ethanol. As long as the carbon-limited feed rate is lower than the capacity for carbohydrate uptake, the cells should respond to changes in the concentration of either substrate merely by increasing the biomass as we have observed in preliminary studies. We will confirm this expectation by introducing a temporally varying stream of mixed sugar into the fermenter, and monitoring how the composition of the culture changes (including the population of each strain). The result of this portion of the research will be a complete and quantitative description of the fermentation of sugar mixtures to ethanol by substrate-selective strains.

Example VII.

Consumption of acetate by *Escherichia coli* without sugar degradation

[0152] A strain of *E. coli* is constructed which consumes acetate but not any of the five principal sugars in biomass hydrolysate as described in Example II. This process will require knockouts of all the sugar uptake systems. We have already demonstrated in Example I that this approach works for xylose and glucose, the two principal sugars in most lignocellulosic hydrolysates.

[0153] The strain(s) used to make these knockouts are those that exhibit high growth rate and high biomass yield on acetate. Tolerance of the organism to high acetate concentration is not important (within a range) because we envision the process operating in fed-batch or continuous mode under carbon (i.e., acetate) limitation. Under these circumstances the concentration of acetate in the fermenter will be maintained at zero and cell "tolerance" to the acetate will not be relevant. However, the rate at which acetate is consumed will directly affect the productivity of the entire process. Therefore, the cells should ideally be able to grow at a high growth rate which will enable the process to run at a high (dilution) rate without any negative consequences.

[0154] Although several research groups have studied growth of *E. coli* on acetate and completed detailed flux analyses, there has not been a comprehensive comparison of the growth rate of *E. coli* strains on acetate as the sole carbon source. We will examine 8-10 diverse wild-type and common strains of *E. coli* (e.g., MG1655, DH5α, MC4100, BL21, JM109, etc.) and grow them as accelerostats (Paalme et al., 1997, Ant. van Leeuwen. 71:217). B strains are preferred because these strains appear to have an elevated expression of enzymes in the glyoxylate shunt (van de Walle and Shiloach, 1998, Biotechnol. Bioeng. 57:71; Phue and Shiloach, 1998, J. Biotechnol. 109:21), which is an important pathway for acetate metabolism. A preliminary study in shake flasks has indicated some strains do indeed grow with a very high rate on acetate (0.70 h^{-1}, about 3 times faster than on MG1655 which was selected for preliminary studies). In this study we determined the growth rate of several publicly available bacterial strains. The table below shows observed growth rates.

	Specific	
	Growth	biomass
Strain	Rate	yield
	(h-1)	(g/g)
AG1	0.676	0.303
MC1061	0.520	0.315
MC4100	0.371	0.279
9637	0.368	0.327
JM101	0.322	0.285
BL21	0.305	0.232
MG1655	0.253	0.309
W3110	0.245	0.281

This approach can be readily used establish biomass yield and maximum growth rate. The two "best" strains, AG1 and MC1061, are selected to knockout the sugar-consuming abilities (described below). Note that the strain found to be the "best acetate consumer" will not necessarily be related to the strain ultimately found to be the "best sugar consumer/ethanol producer." A significant advantage of our process design is that these two strains can be selected independently.

[0155] In order to learn *why* certain strains grow more quickly on acetate, we will complete a genome-wide microarray study. For four of the strains (two "fast growers" and two "slow" growers), we will take samples from our accelerostat experiments (which occurs at a pseudo-steady state) at three different growth rates (approx. 0.1, 0.2, 0.3 h^{-1}). We will conduct microarrays comparing expression at these growth rates (i.e., 0.3 vs. 0.1 and 0.2 vs. 0.1) and at the highest growth rate between the strains (strain 2 vs. strain 1, strain 3 vs. strain 1, strain 4 vs. strain 1). With (independent) triplicate experiments, this will involve 15 microarrays. This approach is similar to our previous study at 6 steady-state growth rates to establish the regulatory importance of the *arcAB* regulatory network in acetate overflow metabolism (Vemuri et al., 2006, Appl. Environ. Microbiol. 72(5):3653).

[0156] Once suitable strains of *E. coli* are identified, they must be made acetate-selective, i.e., they will need deletions in genes involved in the uptake of glucose, mannose, galactose, xylose and arabinose. In the two strains selected for their "best" acetate metabolism, the six genes *manZ, ptsG, glk, xylA, galK, araA* will be knocked out as described in Example IV. None of these genes has any known relationship with acetate consumption, a process which is mediated by acetyl CoA synthase (*acs*), isocitrate lyase (*aceA*), malate synthase (*aceB*) and isocitrate dehydrogenase (*icdA*) (Holms, 1986, Curr Top Cell Regul 28:69).

Example VIII.

Use of simulated and real hydrolysates

[0157] Two-stage fermentations of simulated mixed xylose, glucose, mannose, arabinose, galactose and acetate solutions as well as actual hydrolysates will be conducted using the constructed *E. coli* strains. A "simulated" solution is merely a synthetic (and reproducible) medium prepared with purified components in appropriate proportions to represent a real hydrolysate. In other words, we will prepare a defined medium containing these six compounds as potential carbon sources. Concentrations of these compounds in actual hydrolysates vary considerably (Barbosa et al., 1992, Appl. Environ. Microbiol. 58:1382; Johansson et al., 2001, Appl Environ Microbiol. 67:4249; Taherzadeh et al., 2001, Appl Biochem Biotechnol. 95:45; Brandberg et al., 2004, J. Biosci Bioeng. 98:122), and we will examine a range of concentrations: 20-40 g/L glucose, 5-20 g/L xylose, 1-5 g/L galactose/mannose/arabinose and 2-8 g/L acetic acid. Although we have the ability to generate biomass hydrolysates, we will primarily rely on real hydrolysates obtained from researchers at other Universities and Federal Labs. As necessary, the hydrolysate will be supplemented with other nutrients such as nitrogen, phosphorus and sulfur.

[0158] The initial studies will use simulated hydrolysates. The first stage in the process (the left bioreactor in Fig. 1) involves the removal of acetic acid by feeding an acetate-containing hydrolysate into the reactor so that the organisms grow continuously and the acetate-free stream with cells exits the vessel continuously. Using the growth rate data obtained previously, we will feed the acetate at a dilution rate below the maximum growth rate and scale the process accordingly. We will determine the ranges of acetate concentration in the hydrolysate that are acceptable, and the rates for which the process can be conducted. We will determine the long-term stability of the process. The acetate consumption rate determined from such data will be helpful in sizing a pilot/commercial process. When acetate is the limiting nutrient under aerobic conditions, we anticipate that the carbon in acetate will be converted either into cells or CO_2. We will nevertheless need to complete a detailed carbon balance to account for the utilization of carbon under various operating conditions. The formation of the potential metabolic products such as ethanol, lactate, formate, succinate, and fumarate in the detoxification step is not anticipated but will be determined in addition to the concentrations of the six substrates via chromatography (Eiteman and Chastain, 1997, Anal. Chim. Acta 338:69).

[0159] Additionally, we will demonstrate the robustness of the process by "ramping" the concentration of acetate in the feed and observing the response of the microbial system to changing acetate concentration. We anticipate that the cells will naturally adapt to changing concentrations of acetate in the feed stream by increasing the biomass concentration, as we have observed in preliminary studies for the two-substrate system. For simulated hydrolysate we anticipate that the stream exiting Stage 1 will contain exclusively 5 sugars (and biomass) as carbon sources.

[0160] Stage 2 will be fed the stream exiting Stage 1. It is advantageous to maintain Stage 1 as a continuous (or fed-batch) process to maintain a zero acetate concentration as a result of substrate limitation. The matching of this continuous process with Stage 2 will be a significant aspect of this part of the research project. Three different bioprocess modes will be examined for Stage 2, including: 1) batch, 2) linear fed-batch, and 3) exponential fed-batch. A batch process will be conducted by fermenting a discrete portion of the (continuous) effluent from Stage 1. Such a process will require storing some of the Stage 1 effluent in a tank. A linear fed-batch will be conducted by synchronizing the Stage 1 effluent rate with the feeding rate to Stage 2. In this case, because the biomass concentration in Stage 2 is small the feed initially into Stage 2 will exceed the rate of carbohydrate consumption, and the cells will grow at their maximum growth rate. Later after the biomass concentration increases, the maximum growth rate will exceed the carbohydrate feed rate and Stage 2 will become carbon limited. An exponential fed-batch, accomplished via a programmable pump, will involve holding a portion of the Stage 1 effluent initially and gradually increasing the rate of feeding to match a desired cell growth rate. We routinely perform exponential fed-batch processes (e.g., Smith et al., 2006, Appl. Microbiol. Biotechnol. 28:1695) as they are advantageous to control growth and product formation rates carefully. The focus of this portion of the research will be to study the kinetics of sugar consumption subsequent to acetate removal. Process stability, mode of operation, robustness to varying feed compositions and sensitivity to inoculation approaches will all be addressed in this portion of the project.

[0161] We will study ethanol production in this process, using a single organism process with *E. coli* KO11 (without additional substrate-selectivity) as an experimental control. Although a couple of different ethanol-production approaches are possible depending on the specific strain, we envision a two-step process for Stage 2 wherein the first step is an aerobic growth phase using the two strains and substrates. A second step involves a potential anaerobic production phase wherein all the carbon is directed to the product of interest, ethanol, and growth is low. Growth can be slowed by several means including limiting the feed in another nutrient such as nitrogen (i.e., ammonium ion).

[0162] We will also obtain lignocellulosic hydrolysates and determine the detoxification of that hydrolysate using the acetate-selective strain in the developed process. As noted, it is likely that the raw hydrolysate will require supplementation with additional nutrients in order to permit growth of the acetate-selective strain in Stage 1 and the growth of the 4 sugar-selective strains in Stage 2. Several commercial processes use nutrient supplementation which does not negatively impact the cost of production (ammonia, phosphate). We will readily be able to determine whether the hydrolysate is, for example, phosphate-limited rather than carbon-limited by determining the nutrient levels in the effluent stream. We anticipate that the feasible feed rates for both stages will be lower when lignocellulosic hydrolysate is used compared to simulated hydrolysate. The results from these studies will be critically compared with the results obtained using the simulated xylose, glucose and acetate solution.

Example IX.

Concurrent use of xylose and glucose by Lactic Acid Bacteria

[0163] So-called facultative homofermentative strains of Lactic Acid Bacteria are of great interest because they are able to consume either xylose or glucose (and other sugars), and many also have a high tolerance to low pH and temperatures exceeding 50°C. These qualities make these microorganisms particularly relevant for the production of lactic acid (currently manufactured commercially) and other products including ethanol. The substrate-selective approach described herein can be advantageously utilized in this organism. Strains of *Lactobacillus* and *Lactococcus* which are able to consume selectively xylose and glucose will be constructed. Other researchers have explored ethanol production in *Lactobacillus* (Nichols et al., 2003, J Indust. Microbiol. Biotechnol. 30:315). This study is expected to show that (1) inhibitor-removal and sugar-conversion steps are decoupled in the method described herein, and (2) two different species can be used in the two separate stages without any disadvantages. Importantly, because of these advantages, researchers no longer have to pass over a strain for the production of a biochemical merely because it is intolerant of another chemical which could now be removed (using our approach) in a previous stage by another organism.

[0164] First, isogenic xylose-selective and glucose-selective strains of Lactic Acid Bacteria will be constructed. A Lactic Acid Bacteria strain that can consume both xylose and glucose is chosen as the starting strain for developing the isogenic xylose-selective and glucose-selective strains. Like *E. coli*, Lactic Acid Bacteria possess glucose-specific uptake pathways in addition to the general PTS which facilitates the import of a variety of sugars. The cytoplasmic phosphocarrier enzymes of the PTS have been identified in several Lactic Acid Bacteria species and are encoded by the *ptsH* and *ptsI* genes which reside in an operon, and as in *E. coli,* Lactic Acid Bacteria *ptsHI* deletion mutants are still able to metabolize glucose (Stentz et al., 1997, Appl. Environ. Microbiol. 63:2111; Luesink et al., 1999, J. Bacteriol. 181:764; Viana e al., 2000, Mol. Microbiol. 36:570). However, because the glucokinase homologue has not been identified in a Lactic Acid Bacteria species in which the *ptsHI* operon has also been characterized, we will start with a Lactic Acid Bacteria species in which *ptsHI* has been deleted in order to construct a xylose-selective Lactic Acid Bacteria strain.

[0165] *Lactobacillus casei, Lactobacillus sake,* and *Lactococcus lactis* are species of Lactic Acid Bacteria in which the *ptsHI* genes have been characterized and which also can utilize xylose as a sugar source (Zhang et al., 1995, Appl. Biochem. Biotechnol. 51/52:527; Erlandson et al., 2000, Appl Environ Microbiol. 66:3974). Similar to the *E. coli* studies in which we select a strain for acetate consumption (Example VII), we will first compare these three candidate Lactic Acid Bacteria to determine their growth rates in minimal xylose media to choose a preferred strain for these studies. *ptsHI* derivatives of each of these three strains are available (Stentz et al., 1997, Appl. Environ. Microbiol. 63:2111; Luesink et al., 1999, J. Bacteriol. 181:764; Viana e al., 2000, Mol. Microbiol. 36:570), and a *ptsHI* deletion derivative of the selected strain will be mutagenized in order to generate a Lactic Acid Bacteria that is incapable of growing on minimal glucose media. Both nitrosoguanidine and ethyl methanesulfonate will be employed as mutagens and if necessary antibiotic enrichment procedures and MacConkey Glucose selective plates will be used to identify a Lactic Acid Bacteria *ptsHI* mutant that is xylose-selective and incapable of metabolizing glucose.

[0166] An isogenic glucose-selective Lactic Acid Bacteria strain will then be generated. Like *E. coli,* Lactic Acid Bacteria strains which contain mutations in either the genes that encode for the xylose transporter or isomerase or xylulokinase enzymes cannot utilize xylose as a sole carbon source (Lokman et al., 1991, Mol Gen Genet. 230:161; Chaillou et al., 1998, Appl. Environ.

Microbiol. 64:4720; Erlandson et al., 2000, Appl Environ Microbiol. 66:3974). Chemical mutagenesis will be employed to generate a Lactic Acid Bacteria *xyl* mutant using the same "optimal" strain as described above. The result will be two isogenic strains of a species of Lactic Acid Bacteria, one which is xylose-selective and a second which is glucose-selective.

[0167] Next, fermentations of sugar mixtures will be conducted. Analogous to the more comprehensive research completed for *E. coli* in the above Examples, as part of this experiment with Lactic Acid Bacteria, we will complete fermentations of a medium composed of xylose and glucose using the substrate-selective strains. A unique aspect of many Lactic Acid Bacteria is their tendency to switch between heterofermentative and homofermentative conditions as a function of growth rate and redox conditions (Garrigues et al., 1997, J. Bacteriol. 179:5282). We will study both low growth rates at which mixed-acid (and ethanol) production is expected and high growth rates at which lactic acid production dominates. Previous difficulties with ethanol production in Lactic Acid Bacteria (Nichols et al., 2003, J. Indust. Microbiol. Biotechnol. 30:315) may be attributed to the competition between lactic acid formation and ethanol formation under high growth, homofermentative conditions. Although the focus will be the simultaneous utilization of xylose and glucose and not on product formation, per se, an understanding of product formation and its relationship to operational parameters is important in the interpretation and analysis of the results.

[0168] We will conduct chemostat experiments to establish the operating ranges for the processes and the performance under both homofermentative and heterofermentative conditions. We will determine for each strain the biomass yields, specific rates of consumption/production of dissolved and gaseous compounds, and the maintenance energy requirements resulting from the various genetic perturbations. We will study the strains individually on single-substrate media, then single strains on dual-substrate media, then multiple strains on dual-substrate media. We will assess whether the presence of the unutilized sugar impacts the ability of the strain to consume its exclusive substrate, determine the operating optimal conditions and ranges for the conversion process, and examine how the distribution of end-products is affected by the co-culture. These experiments will facilitate the development of future processes to convert lignocellulosic hydrolysates efficiently into useful products with other organisms such as yeast.

Example X.

Substrate-selective *Saccharomyces cerevisiae*

[0169] Because yields and productivity are the two most important economic factors in the manufacture of fuel ethanol, the preferred microorganism of choice is Saccharomyces cerevisiae. This yeast ferments ethanol very efficiently and can tolerate the highest ethanol concentrations of any known microorganism. Unfortunately, the use of *S. cerevisiae* to produce fuel ethanol from lignocellulosic hydrolysates is problematic, because it cannot utilize the pentose sugars xylose and arabinose which are two of the most abundant sugars that are found in lignocellulosic hydrolysates. Researchers have attempted to solve this problem by constructing new *S. cerevisiae* derivatives that contain the genes required for xylose utilization. These new yeast derivatives can utilize xylose, however, when these yeasts are fed a mixed carbon source that contain glucose and xylose, the glucose must be consumed first, before any xylose can be utilized.

[0170] Our multiple cell approach can be readily applied to *S. cerevisiae*. *S. cerevisiae* strains that cannot utilize galactose can be constructed by deleting the GAL2, GAL1, GAL7, or GAL10 genes. These strains will be able to utilize glucose as a carbon source. *S. cerevisiae* strains that cannot utilize glucose can be constructed by deleting the GLK1, HXK1, and HXK2 genes or the HXT1, HXT2, HXT3, HXT4, HXT6, HXT7, and SNF3 genes. These strains will be able to utilize galactose as a carbon source. *S. cerevisiae* strains that can utilize xylose or arabinose can be constructed by importing these pathways from other yeasts such as *Pichia stipitis* or *Ambrosiozyma monospora* which are able to utilize these sugars. By knocking out the genes that are required for glucose and galactose utilization in *S. cerevisiae* strains that have been genetically modified to consume xylose and/or arabinose, new strains can be created which are *only* able to utilize xylose and/or arabinose. Concurrent use of a combination of these strains would be able to produce ethanol very efficiently.

Example XI.

Formation of lactate by simultaneous consumption of xylose and glucose in an *E. coli* co-fermentation

[0171] We report a new approach for the simultaneous conversion of xylose and glucose sugar mixtures into lactic acid. The process simultaneously uses two substrate-selective strains of *Escherichia coli,* one which is unable to consume glucose and one which is unable to consume xylose. In addition to knockouts in *pflB* encoding for pyruvate formate lyase, the xylose-selective (glucose deficient) strain *E. coli* ALS1073 has mutations in the *glk, ptsG* and *manZ* genes while the glucose-selective (xylose deficient) strain *E. coli* ALS1074 has a mutation in the *xylA* gene. By combining these two strains in a single process having this sugar mixture, xylose and glucose are simultaneously converted to lactate. This approach to the utilization of mixed sugars eliminates the problem of diauxic growth, and provides great operational flexibility.

[0172] Recently, we proposed a multi-organism approach to the problem of utilizing sugar mixture (Eiteman et al., J Biol Eng 2008, 2:3; Example I). This approach involves introducing strains into a substrate mixture which are each unable to consume all but one particular substrate. Therefore, each strain will ignore other substrates while it stably carries out the desired conversion of that one selective substrate. One advantage of such "substrate-selective uptake" is that the system can adapt to fluctuations in the feed stream; that is, cultures can grow in concert with a variable feed composition. Also, metabolic engineering strategies could ultimately focus on improving the individual production strains independently. For example, the glucose-selective strain could be improved for the generation of a particular product without concern for the impact of these changes on the conversion of xylose to that product-one objective does not have to be compromised for another.

[0173] In this example, we use substrate-selective strains of *Escherichia coli* for the formation of lactic acid in a mixed-sugar defined medium. Over 1.5M lactate can be generated by an organism having several key mutations under carefully controlled conditions (Zhu et al., 2007, Appl Environ Microbiol 73:456). We use strains having just one additional mutation in central metabolism to demonstrate the feasibility of a two-strain strategy for the conversion of xylose and glucose into lactate.

Methods

Strains

[0174] *Escherichia coli* strains ALS1073 (*xylA pflB*::Cam) and ALS1074 (*ptsG manZ glk pflB*::Cam) were derived from MG1655 (wild-type, F-, λ-). In a batch culture, ALS1073 will not consume xylose, while ALS1074 will not consume glucose. The *pflB* mutation prevents growth under anaerobic conditions, but causes the accumulation of significant lactate (de Graef et al., 1999, J Bacteriol 181:2351).

Growth conditions

[0175] For each bioreactor experiment, a single strain was first grown in a tube containing 10 mL B2X3G medium, then 5 mL transferred to 50 mL B2X3G medium in a 250 mL shake flask. All flasks were incubated at 37°C and 250 rpm (19 mm pitch). For those fermentations in which a single strain was used, when the OD of the shake flask culture reached approximately 4, the contents of the shake flask were diluted with B2X3G medium so that 100 mL having an effective OD of 2.0 was used to inoculate the fermenter. For those experiments in which two strains were used in a single process, the contents of two shake flasks were diluted with B2X3G medium to 100 mL so that each strain had an effective OD of 2.0 (i.e., in the 100 mL volume). Basal medium contained (per L): 13.3 g KH_2PO_4, 4.0 g $(NH_4)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$, 13.0 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1.5 mg $CuCl_2 \cdot 2H_2O$, 15.0 mg $MnCl_2 \cdot 4H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 100 mg Fe(III)citrate, 8.4 mg $Na_2EDTA \cdot 2H_2O$, 1.7 g citric acid, and 0.0045 g thiamine·HCl. B2X3G medium comprised basal medium with 20 g/L xylose and 30 g/L glucose. The shake flask medium was adjusted to a pH of 7.0 with 20% NaOH.

Fermentation

[0176] Batch experiments were carried out in a 2.5 L bioreactor (Bioflow 2000, New Brunswick Scientific Co. Edison, NJ, USA) containing 1.0 L BXG medium. Each process consisted of two phases maintained at 37°C. During an aerobic growth phase, air was sparged into the fermenter at a flowrate of 1.0 L/min, and the agitation was 1000 rpm to ensure no oxygen limitation. The pH was controlled at 6.7 using 15% (w/v) NH_4OH. After 8 h anaerobic conditions commenced, N_2 at 0.2 L/min was supplied, the agitation reduced to 200 rpm, and the pH was controlled at 6.7 using 20% (w/v) NaOH.

Analyses

[0177] The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth, and this value was correlated to dry cell mass. Previously described liquid chromatography methods were used to quantify xylose and glucose (Eiteman and Chastain, 1997, Anal Chem Acta 338:69) and other organic compounds (Chesson et al., 1993, J Sci Food Agric 34:1330).

Results

[0178] *Escherichia coli* ALS1074 and ALS1073 are unable to consume glucose and xylose, respectively. The xylose-selective strain ALS1074 has mutations in the three genes involved in glucose uptake (Curtis and Epstein, 1975, J Bacteriol 122:1189), rendering it unable to consume glucose: *ptsG* codes for the Enzyme $IICB^{Glc}$ of the phosphotransferase system (PTS) for carbohydrate transport (Postma et al., 1993, Microbiol Rev 57:543), *manZ* codes for the IID^{Man} domain of the mannose PTS permease (Huber and Erni, 1996, Eur J Biochem 239:810), *glk* codes for glucokinase (Curtis and Epstein, 1975, J Bacteriol 122:1189). ALS1073 has a knockout in the *xylA* gene encoding for xylose isomerase, rendering ALS1073 unable to consume xylose. In a medium composed of a mixture of these two sugars, ALS1074 would be expected to consume the xylose selectively while ALS1073 should exclusively consume the glucose. Each strain also has a mutation in *pflB* encoding for pyruvate formate lyase. This mutation under anaerobic conditions creates a severe metabolic bottleneck at pyruvate, curtailing growth in the absence of acetate and diverting most carbon to lactate (de Graef et al., 1999, J Bacteriol 181:2351).

[0179] In our first experiment, we grew individually ALS1073 or ALS1074 under aerobic conditions, and after 8 h switched to anaerobic conditions. ALS1073 consumed approximately 10 g/L glucose during the 8 h of growth to an OD of 11.5 (Fig. 10). After commencing anaerobic conditions, growth stopped and the remaining 17 g/L glucose was converted into about 14 g/L lactate within 3 h for a yield of 0.83 g/g. About 1.5 g/L succinate and less than 0.5 g/L acetate and ethanol as by-products were also generated during the anaerobic phase. Lactate was formed at a constant specific rate of formation of about 1.2 g/g·h during the anaerobic phase. During the two phases of growth and product formation, the xylose concentration remained unchanged.

[0180] ALS1074 consumed 4 g/L xylose during the 8 h aerobic growth phase to an OD of 4.5 (Fig. 11). During the anaerobic phase, the remaining 13.3 g/L xylose was converted to 13 g/L lactate at a yield near 1.0 g/g. The conversion of xylose to lactate was completed after 14 h. The rate of xylose consumption by ALS1074 appears slower than observed for the rate of glucose consumption by ALS1073 (shown in Fig. 10). However, this observation must be considered in light of the significant difference in cell biomass concentration between the two experiments-the cell concentration for the xylose-only consumption using ALS1074 was 40% of the concentration for glucose-only consumption using ALS1073. The specific rate of xylose consumption was 0.92 g/g·h at the onset of the anaerobic phase, whereas the rate of xylose consumption was 0.49 g/g·h during the latter portion of the anaerobic phase. Less than 0.5 g/L succinate and no acetate and ethanol were generated as by-products, and the glucose concentration remained unchanged during the entire process.

[0181] In a second experiment, *both* ALS1073 and ALS1074 were inoculated into a single bioreactor containing the xylose-glucose defined medium. In this two-strain co-fermentation, care was taken to ensure that the inoculum consisted of each microorganism at the same concentration as had been inoculated in the one strain processes. After 8 h of growth, the culture had consumed about 4 g/L xylose and 10 g/L glucose to achieve an OD of 16 (Fig. 12). During the subsequent anaerobic phase, the remaining glucose was consumed in less than 3 h, and the xylose was consumed in about 12 h. Assuming that the OD of 16 represents an OD of 11.5 for ALS1073 and an OD of 4.5 for ALS1074 (values observed in the previous single organism cases), then the specific rate of glucose consumption during the anaerobic phase was 1.6 g/g·h, and the rate of xylose consumption was initially 1.2 g/g·h. The 17.5 g/L glucose and 13.3 g/L xylose present at the onset of the anaerobic phase were converted to 25.9 g/L lactate, for a combined yield of 0.84 g/g. About 2 g/L succinate and less than 0.5 g/L acetate and ethanol were generated as by-products. The experiment demonstrates that the two strains acted independently in the conversion of xylose and glucose to lactate.

Discussion

[0182] The process described in this study demonstrates the simultaneous conversion of sugar mixtures into one particular product: lactate. In this case, two strains were used which are each selective in their consumption of the two carbon sources present, xylose and glucose. Excluding substrate consumption in a strain by gene deletions represents a new approach for the conversion of multiple substrates into a desired product. If additional carbon sources were present, then additional mutations and strains would be necessary to ensure each strain exclusively converted one substrate into the desired product.

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Fremgangsmåde til fremstilling af et biokemikalie, hvilken fremgangsmåde omfatter samtidig etablering af kontakt mellem et lignocelluloseholdigt hydrolysat og (a) en flerhed af genmodificerede sukkerselektive Escherichia coli-celler; eller (b) en flerhed af genmodificerede sukkerselektive Saccharomyces cerevisiae-celler; hvor flerheden af sukkerselektive celler omfatter sukkerselektive første- og andenceller, hvor den første sukkerselektive celle metaboliserer en første sukker, som ikke kan metaboliseres af den anden sukkerselektive celle, og den anden sukkerselektive celle metaboliserer en anden sukker, som ikke kan metaboliseres af den første sukkerselektive celle, under betingelser, som gør det muligt for flerheden af genmodificerede sukkerselektive celler at fremstille biokemikaliet; hvor den første og den anden sukker er uafhængigt valgt fra gruppen bestående af glucose, xylose, arabinose og galactose.

2. Fremgangsmåde ifølge krav 1, som endvidere omfatter oprensning af biokemikaliet.

3. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor biokemikaliet er valgt fra gruppen bestående af ethanol, butanol, succinat, lactat, fumarat, pyruvat, smørsyre og acetone.

4. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor biokemikaliet er ethanol, og hvor mindst én af flerheden af sukkerselektive celler er blevet yderligere genmodificeret til forøget ethanolfremstilling.

5. Sammensætning, som omfatter (a) en flerhed af genmodificerede sukkerselektive E. Coli-celler eller (b) en flerhed af genmodificerede sukkerselektive S. cerevisiae-celler, hvor flerheden af genmodificerede sukkerselektive celler omfatter sukkerselektive første- og andenceller, hvor

den første sukkerselektive celle metaboliserer en første sukker, som ikke kan metaboliseres af den anden sukkerselektive celle, og den anden sukkerselektive celle metaboliserer en anden sukker, som ikke kan metaboliseres af den første sukkerselektive celle, hvor den første og den anden sukker er uafhængigt valgt fra gruppen bestående af glucose, xylose, arabinose og galactose.

6. Sammensætning ifølge krav 5, som endvidere omfatter et lignocelluloseholdigt hydrolysat.

7. Sammensætning ifølge krav 5 eller 6, som endvidere omfatter mindst én hæmmerselektiv E. coli-celle, hvor den hæmmerselektive celle er en acetatselektiv celle.

8. Fremgangsmåde eller sammensætning ifølge et hvilket som helst af de foregående krav, hvor flerheden af sukkerselektive celler omfatter mindst én hexoseselektiv celle og mindst én pentoseselektiv celle.

9. Fremgangsmåde eller sammensætning ifølge et hvilket som helst af de foregående krav, hvor mindst én af flerheden af sukkerselektive celler ikke kan metabolisere mindst to sukkere, som er uafhængigt valgt fra gruppen bestående af glucose, xylose, arabinose og galactose.

10. Fremgangsmåde eller sammensætning ifølge et hvilket som helst af de foregående krav, hvor mindst én af flerheden af sukkerselektive celler kun kan metabolisere én sukker valgt fra gruppen bestående af glucose, xylose, arabinose og galactose.

11. Fremgangsmåde eller sammensætning ifølge et hvilket som helst af de foregående krav, hvor flerheden af sukkerselektive celler omfatter:
en første celle, som omfatter en modifikation eller deletion af mindst ét gen, som er involveret i glucosemetabolisme, for således at forhindre den første celle i at forbruge glucose;

og
en anden celle, som omfatter en modifikation eller deletion af mindst ét gen, som er involveret i xylosemetabolisme, for således at forhindre den anden celle i at forbruge xylose.

12. Fremgangsmåde eller sammensætning ifølge et hvilket som helst af de foregående krav, hvor mindst én af flerheden af sukkerselektive celler er blevet genmodificeret til forøget ethanolfremstilling.

13. Fremgangsmåde ifølge et hvilket som helst af kravene 1-4 og 8-12, som endvidere omfatter etablering af kontakt mellem det lignocelluloseholdige hydrolysat og mindst én hæmmerselektiv E. coli-celle under betingelser, som gør det muligt for den hæmmerselektive celle at metabolisere hæmmeren, hvor den hæmmerselektive celle er en acetatselektiv celle.

14. Fremgangsmåde ifølge krav 13, hvor etableringen af kontakt mellem det lignocelluloseholdige hydrolysat og mindst én hæmmerselektiv celle sker forud for eller samtidig med etablering af kontakt mellem det lignocelluloseholdige hydrolysat og flerheden af sukkerselektive celler.

15. Fremgangsmåde ifølge krav 14, som omfatter:
et første trin, som omfatter etablering af kontakt mellem det lignocelluloseholdige hydrolysat og mindst én hæmmerselektiv celle under betingelser, som gør det muligt for den hæmmerselektive celle at metabolisere hæmmeren, for at opnå et afgiftet hydrolysat; og
et andet trin, som omfatter etablering af kontakt mellem det afgiftede hydrolysat og flerheden af sukkerselektive celler for at fremstille biokemikaliet;
hvor den hæmmerselektive celle er en acetatselektiv celle.

16. Fremgangsmåde eller sammensætning ifølge et hvilket som helst af kravene 7 eller 13-15, hvor den hæmmerselektive celle omfatter en modifikation eller deletion af mindst ét gen, som er involveret i glucosemetabolisme, for således at forhindre

cellen i at forbruge glucose, og en modifikation eller deletion af mindst ét gen, som er involveret i xylosemetabolisme, for således at forhindre cellen i at forbruge xylose.

17. Fremgangsmåde eller sammensætning ifølge et hvilket som helst af kravene 7 eller 13-16, hvor den hæmmerselektive celle er blevet genmodificeret således, at den ikke kan metabolisere nogen sukker valgt fra gruppen bestående af glucose, xylose, arabinose og galactose.

18. Sammensætning, som omfatter (a) en flerhed af genmodificerede sukkerselektive E. coli-celler eller (b) en flerhed af genmodificerede sukkerselektive S. cerevisiae-celler; hvor hver cellestamme i flerheden af genmodificerede sukkerselektive celler uafhængigt udnytter kun én sukker, som er til stede i et lignocelluloseholdigt hydrolysat.

19. Genmodificeret E. coli-celle, hvor mindst ét gen, som er involveret i glucosemetabolisme, er blevet modificeret eller deleteret for således at forhindre cellen i at forbruge glucose, og mindst ét gen, som er involveret i xylosemetabolisme, er blevet modificeret eller deleteret for således at forhindre cellen i at forbruge xylose.

DRAWINGS

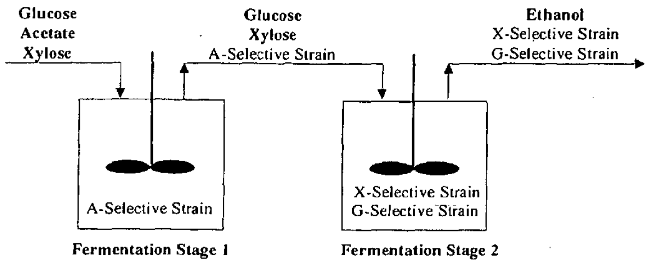


Fig. 1

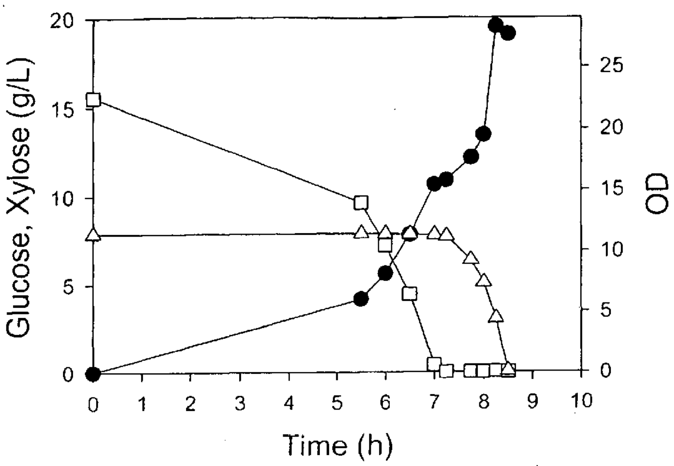


Fig. 2

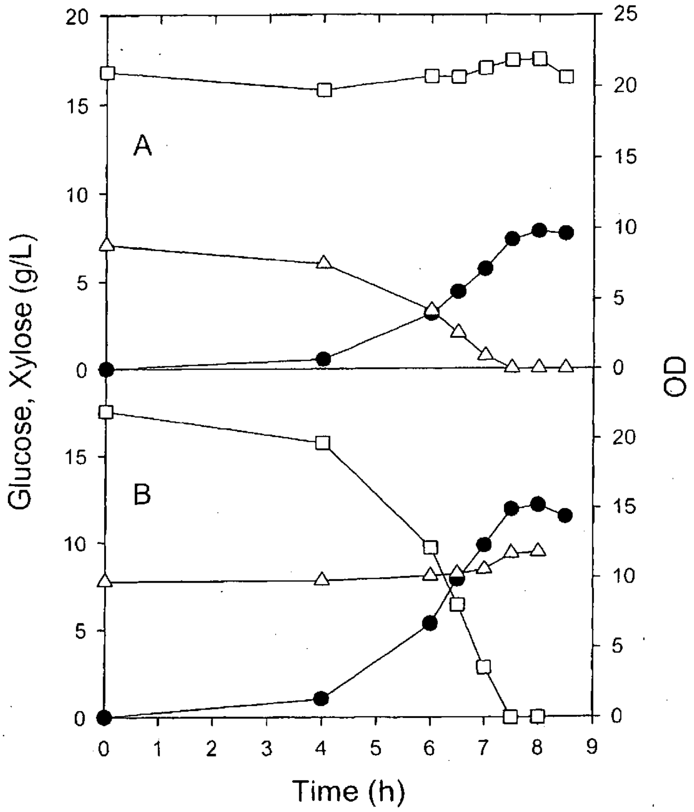

20
15
10
5
0
15
10
5
0
25
20
15
10
5
0
20
15
10
5
0
A
B
Glucose, Xylose (g/L)
OD
0 1 2 3 4 5 6 7 8 9
Time (h)

Fig. 3

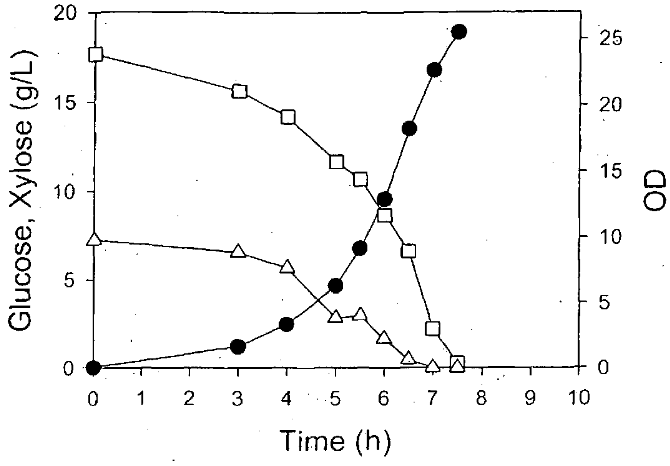


Fig. 4

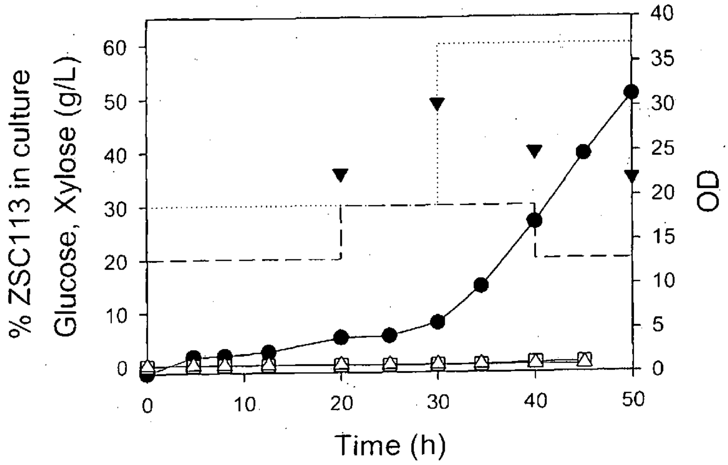

% ZSC113 in culture
Glucose, Xylose (g/L)
60
50
40
30
20
10
0
0
10
20
30
40
50
Time (h)
40
35
30
25
20
15
10
5
0
OD

Fig. 5

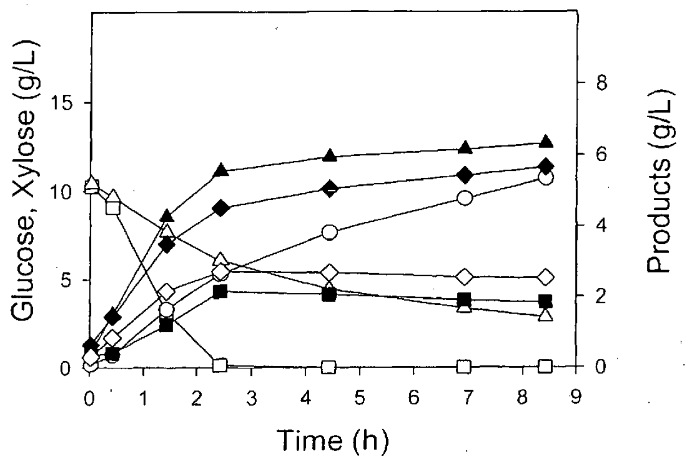

Glucose, Xylose (g/L)
15
10
5
0
Products (g/L)
8
6
4
2
0
0
1
2
3
4
5
6
7
8
9
Time (h)

Fig. 6

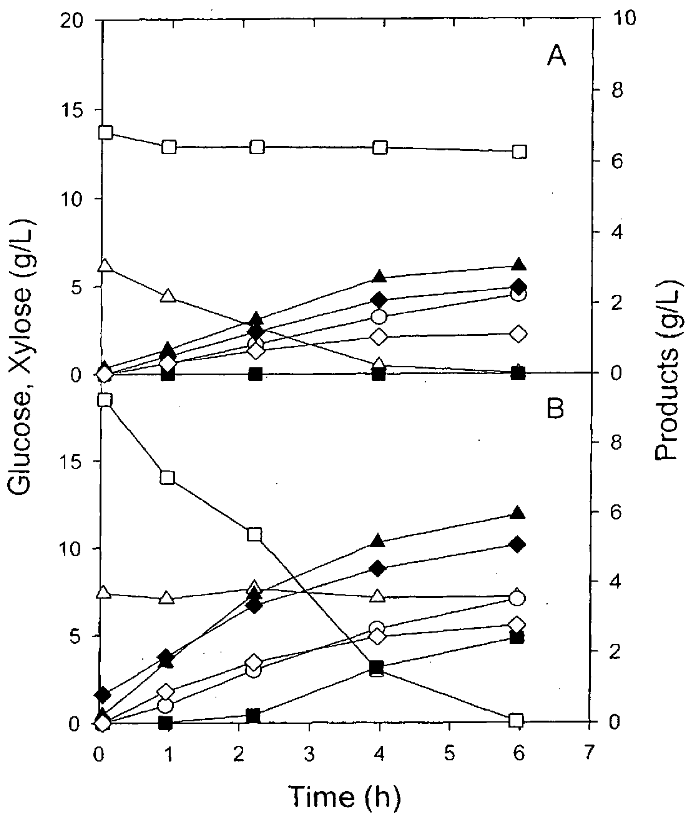

A
B
Glucose, Xylose (g/L)
Products (g/L)
Time (h)
0
5
10
15
20
0
2
4
6
8
10
0
1
2
3
4
5
6
7

Fig. 7

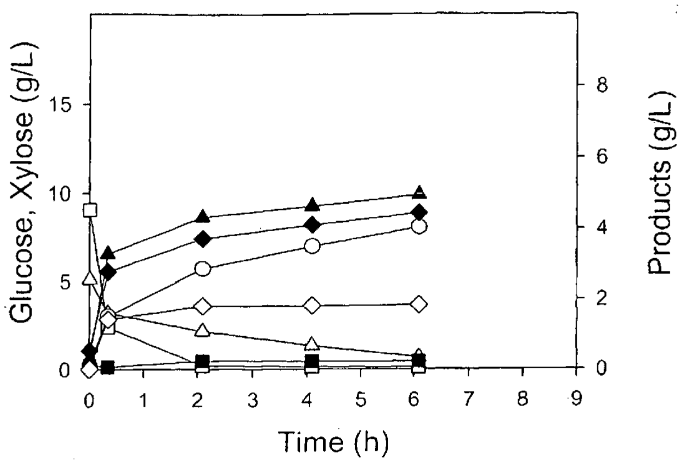

Glucose, Xylose (g/L)
Products (g/L)
Time (h)
0
5
10
15
0
2
4
6
8
0
1
2
3
4
5
6
7
8
9

Fig. 8

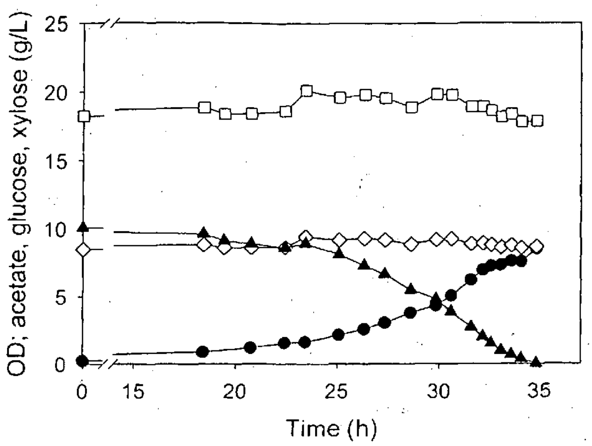

OD; acetate, glucose, xylose (g/L)
25
20
15
10
5
0
0
15
20
25
30
35
Time (h)

Fig. 9

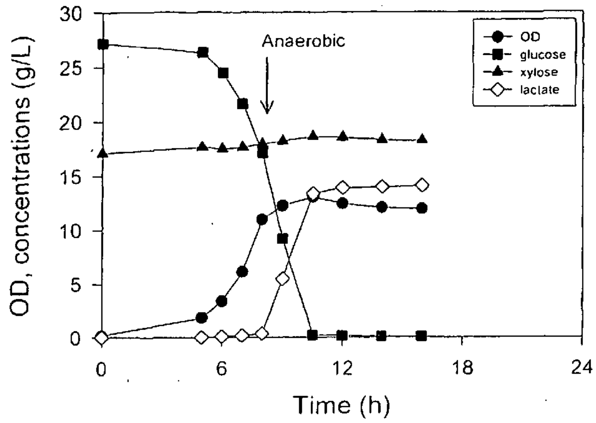

30
25
20
15
10
5
0
OD, concentrations (g/L)
Anaerobic
OD
glucose
xylose
lactate
0
6
12
18
24
Time (h)

Fig. 10

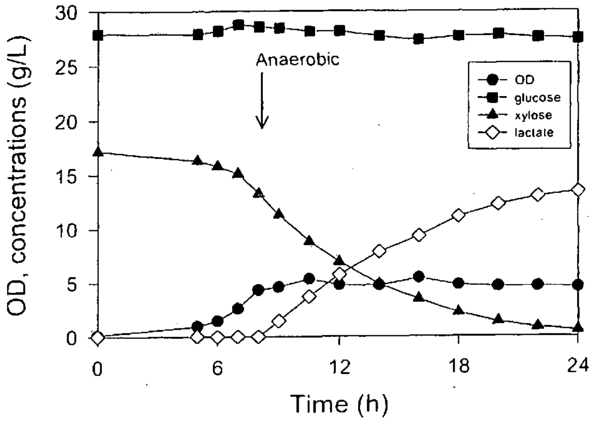
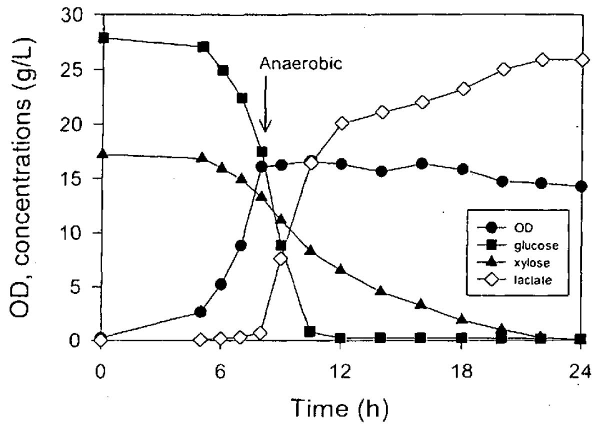

30
25
20
15
10
5
0
OD, concentrations (g/L)
Anaerobic
OD
glucose
xylose
lactate
0
6
12
18
24
Time (h)

Fig. 11



30
25
20
15
10
5
0
OD, concentrations (g/L)
Anaerobic
OD
glucose
xylose
lactate
0
6
12
18
24
Time (h)

Fig. 12