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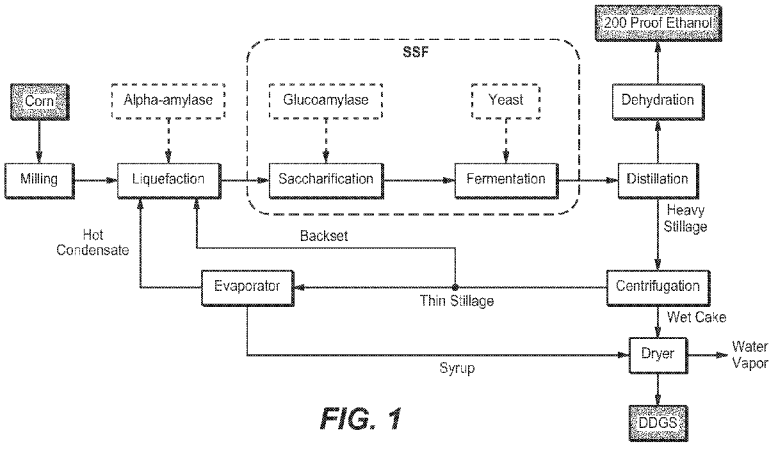

FIG. 1

(57) Abstract: Described are compositions and methods comprising the use of hemicellulases at a level above a certain threshold to improve and increase the production yield of ethanol from grains.

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USE OF HEMICELLULASES TO IMPROVE ETHANOL PRODUCTION

CROSS REFERENCE TO RELATED APPLICATION

[01] This application claims benefit of priority from US provisional application USSN 61/892,299, filed 17 October 2013 and is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[02] Described are methods pertaining to the use of hemicellulases and enzyme mixtures comprising such hemicellulases during saccharification of grain-based plant materials, which leads to the improved production of ethanol from such plant materials and/or the production of by-products having improved properties.

BACKGROUND

[03] Today United States is the largest ethanol producer, and most of that ethanol is blended with gasoline as an oxygenate, at a 10% level typically in the past, but at a higher 15% level in recent years.

[04] Currently most of the fuel ethanol is produced from grain-based feedstock (such as corn), with an insignificant portion, if any, of fuel ethanol produced strictly from cellulosic feedstocks (such as agricultural wastes, switchgrass, other plant materials such as wood, sugar cane, bagasse). While there has been recent and intense interest in producing ethanol from primarily cellulosic feedstocks, most fuel ethanol remains produced in conventional grain ethanol plants today.

[05] Starch is the major carbohydrate storage product in corn kernels, constituting about 70-72% of the kernel weight on a dry weight basis. Starch is readily converted to glucose, using known enzymes, and the glucose thus-made can be fermented into ethanol using a suitable ethanologen, which is typically a yeast.

[06] Two alternative processes, dry grind and wet mill, are used to produce fuel ethanol from grains, each with somewhat different levels of effectiveness, but very different levels of energy and water consumption. The dry grind process is practiced in about two thirds of the grain ethanol industry, whereas the wet mill process is practiced in the remainder. The levels of conversion are not very different between the two processes, with wet mill typically achieving the production of about 2.5 gallons of ethanol per bushel of corn, whereas dry grind achieving the production of a slightly higher number, about 2.8, of gallons of ethanol per bushel of corn.

[07] The energy and capital requirements, on the other hand, are quite substantially different between the wet mill and the dry grind processes. The wet milling process involves separating the grain into starch, fiber, gluten and germ, but higher value products are made from each of the components. For example, after the germ is removed from the kernel, corn oil can be extracted from the germ, at a level of about 1.5 pounds per bushel of corn. The residual germ meal can be added to the corn fiber and hull, forming a corn gluten feed for animals, at a level of about 13.5 pounds per bushel of corn. Gluten can be separated from the rest and be readily processed into corn gluten meal, which is a high-protein premier animal feed, at a level of about 2.5 pounds per bushel of corn. The remaining starch, once separated from the solids, which are used for the various purposes described above, can be made into fermentable sugars, which can in turn be fermented into ethanol.

[08] The dry grind ethanol process, also called dry milling process, processes whole kernels without the steeping and fraction steps. The whole grain is milled, cooked, hydrolyzed, and fermented to produce ethanol. The dry grind process generates less high-value products such as gluten meals or corn oil, but the simpler process also means lesser energy and capital intensity. The clean corn is milled (ground) and mixed with water to form a mash, which is then cooked. Enzymes are added to the cooked mash, converting the starch to fermentable sugars. Yeast is then used to ferment the sugars into ethanol. The mixture containing the ethanol and the remaining solids is then distilled and dehydrated, producing fuel ethanol. The post-distillation solids are dried to produce distillers dried grains, a highly nutritional co-product or by-product that contains differing levels of proteins, unhydrolyzed and unfermented components as well as yeasts at the end of fermentation, which can be used as animal feed supplements, at a level of 15 pounds or more per bushel of corn.

[09] In the dry grind process, after distillation, what remains is a fraction including various solids and a liquid phase that is intermingled with the solids. The fraction includes oil, protein components, but mostly fiber, together with the unfermented starch. It is subject to a separation procedure, where the thin stillage (the liquid part) is separated from the insoluble solid fractions using centrifugation or press/extrusion. The stillage leaving the beer column is centrifuged with a decanter. Often a portion of at least 10% (e.g., 20% or even 30%) of the thin stillage is recycled as backset. The remaining thin stillage is concentrated through evaporation, the resulting concentrated thin stillage, which is now a thick, viscous syrup, is then mixed together with the solid fraction, creating a wet distiller's grain with soluble (WDGS), which can be used directly as an animal feed, but, is often further dried to improve shelf life to allow for the duration of transportation and storage. The furthered dried animal feed product is called dry

distiller's grain with soluble (DDGS). The drying process further adds to the energy consumption, which can often be as high as one third of all energy consumption of a given dry grind plant.

[10] Corn ethanol plants using conventional enzymatic starch processing in either wet milling or dry grind processes will invariably see the loss of theoretical ethanol yield as a result of residual starch. It is believed that at least some of the residual starch is trapped by the cellulosic and hemicellulosic components of the grain material. Therefore reducing or disruption of such entrapment can make previously unavailable starch accessible for fermentation to make more ethanol. To accomplish such disruptions, cellulases and certain hemicellulases have been applied, which were thought to not only help release the residual starch but also potentially increase the production of monomeric and fermentable sugars from the not insignificant amounts of lignocellulosic components of the grain substrates.

[11] Due to its high content of polymeric sugars that have not been utilized during the dry grind process, distillers' grains (DG) have potential not only as an animal feed, but also as an additional source of fermentable sugars to produce more ethanol. The recycle and hydrolysis of distillers' grain benefit dry grind ethanol process by improving the ethanol yield without the need to significantly alter or modify the existing dry grind technology and infrastructure. In practice, DDGs have been mixed with water to achieve an acceptable level of dry solids loading, and the mixture was then pretreated by heating, followed by treatment by a formulation of cellulase such as those commercially available and readily obtained including Spezyme CP (Genencor, Palo Alto, CA), supplemented with beta-glucosidase such as Novozyme 188 (Novozymes, Franklin SC), etc. *Kim et al.,* (2008) Bioresour. Technol. 99, 5206. Other measures aimed at harnessing residual sugars from the DGs include the treatment of such materials with certain hemicellulases such as xylanase, and/or feruloyesterases in addition to cellulase mixtures as those described in *Kim et al.* (*supra*), thereby increasing xylose and arabinose yields.

[12] The DG recycling processes as described above help to increase the overall ethanol yield from a given amount of a grain-based substrate. These processes typically comprised adding cellulases and/or hemicellulases after the initial round of dry grind grain-to-ethanol process. The cellulases and/or hemicellulases are added in a second or follow-on saccharification step in order to break down the cellulosic components of the residual materials.

[13] Entrapped residual starch can also be released upstream prior to or during the saccharification step, by adding cellulass, thereby consolidating the saccharification steps into one. For example, cellulases have been added to the liquefaction step, and to the first and only

saccharification step of a traditional dry grind ethanol production process, which is optionally carried out in conjunction or separately with the fermentation step to enhance the production yield of ethanol. See, for example, the disclosures of U.S. Published Patent Application 2012/0276593.

[14] It is thought that approaches such as these use cellulases to achieve a few potentially interconnected effects: (1) to further reduce the viscosity of the feedstock slurry during liquefaction; (2) to potentially break down the fibrous cellulosic materials in the feedstock slurry releasing soluble cellulosic sugars such as glucose and oligomers that are fermentable to ethanol; and (3) and help to release the residual starch that is entrapped or bound to the cellulosic materials. *See, Ohgren et al.,* (2007) Process Biochemistry, Vol. 42, pp 834-39. Cellulases blended with glucoamylase during saccharfication and/or fermentation has been reported to reproducibly and reliably increase ethanol yield to a small but economically not insignificant amount.

[15] It was said that, if fully converted to glucose, the cellulose of the corn grain can theoretically produce about an additional 0.1 gallon of ethanol per bushel of corn. *Saville & Yacyshyn,* (2005) Effect of Cellulase Supplementation on Cookline Operation in A dry Mill Ethanol Plant, 27th Symposium on Biotechnology for Fuels and Chemicals, May 1-4, 2005, Denver Colo. It was thought that if such a theoretical conversion yield could be achieved, then product yields (of ethanol) can be increased by 0.4 to 0.5% from that obtained from a traditional dry grind process without the addition/inclusion of cellulases. Due to the size of the grain ethanol industry, processes that can improve yields of the fermentation products, such as ethanol, and/or the quality or property of the by-product, would represent an advancement in the art. Even small reproducible improvements in yield, if attainable without additional energy and resource input would be valuable.

SUMMARY

[16] Described is a new method for increasing or improving the level of ethanol produced from a starch-containing grain such as corn kernels by adding treating the liquefact of such a starch-containing substrate with an enzyme composition comprising at or above a threshold level of one or more hemicellulases.

[17] As observed in the prior art, adding cellulases to the saccharification step in conjunction with glucoamylase either breaks down the cellulose of the grain materials in the liquefact, or helps to release the residual starch trapped in the cellulose-part of the grain material, for example the corn fiber, or both. The amount of cellulose in a given grain-based substrate can be readily

determined and it was known that adding increasing amounts of cellulases up to the level of full theoretical conversion of all the cellulose would cause an attendant increase in ethanol yield that is small but significant for commercial purposes.

[18] It is also known that the grain-based materials contain substantial amounts of hemicelluloses, and depending on the type of grain, the hemicellulose content may be greater or smaller. It can be expected that adding hemicellulase(s) to a saccharification step, optionally together with cellulases, in the presence of a glucoamylase, would increase the overall yield of ethanol even further. This, together with the knowledge that, at least when used in the context of cellulosic hydrolysis of lignocellulosic biomass, the presence of hemicellases synergistically boosts the performance of cellulosic conversion. However, quite unexpectedly, when small amount of hemicellulases was added to the saccharification step, not only was there no increase of ethanol yield observed but the process had to be terminated due to significant rise of pressure downstream. A dramatic increase in viscosity or thickness was observed in the post distillation mixture and the thin stillage. The thin stillage became difficult to pump. This made the thin stillage much more difficult to effectively evaporate in order to produce syrup. The increase in thin stillage viscosity and/or thickness can be alleviated by the addition of water to the thin stillage itself, but this approach presents more problems rather than a viable solution because the amount of energy expenditures associated with evaporating and concentration a thus-produced much larger volume of thin stillage is significant, and the resulting syrup would tend to be dilute, difficult to handle, and had much reduced nutritional value.

[19] It was surprisingly discovered that, rather than removing the hemicellulases or reducing the level of hemicellulases in the saccharification to reduce the downstream pressure and viscosity of the thin stillage, thus foregoing the small theoretical increase in ethanol yield that should occur as a result of the hemicellulosic hydrolysis, increasing the level of hemicellulases in the saccharification mixture to above a certain threshold level would resolve not only the downstream pressure buildup issues but also allow the production of an increased overall yield of ethanol.

[20] While not wishing to be bound by theory, it is believed that the viscosity buildup in the thin stillage is caused by the incompletely broken down arabinoxylan oligomers, which are viscose themselves, have high affinity for water and capable of sequestering water. The stepping up or increasing to above a threshold level of hemicellulases in the saccharification step results in a more complete breakdown of the arabinoxylan oligomers into smaller soluble sugars or those sugars with oligosaccharide chains too short to sequester water, thus releasing the sequestered water as well as the remaining trapped starch for ethanol production. The increasing

hemicellulases break down hemicellulose, and in the presence of cellulases, provide a boost to the cellulase activities causing more effective and complete breakdown of cellulose in the grain materials. The increasing hemicellulases as such can also effectuate further release of trapped residual starch.

[21] In a first aspect of the invention, a composition comprising a liquefied starch slurry, a glucoamylase, and one or more hemicellulases is provided. The one or more hemicellulases in the composition of the first aspect are present in at least 0.1 mg/g, for example, at least 0.2 mg/g, at least 0.3 mg/g, at least 0.4 mg/g, at least 0.5 mg/g, at least 0.6 mg/g, at least 0.7 mg/g, at least 0.8 mg/g, at least 0.9 mg/g, at least 1 mg/g, at least 1.2 mg/g, at least 1.5 mg/g, at least 1.8 mg/g, at least 1.9 mg/g, at least 2.0 mg/g, or at least 2.2 mg/g, or even at least 2.5 mg/g of dry solids in the saccharification mixture.

[22] In some embodiments, considering the theoretical level of hemicellulose in a given grain-based substrate available for hydrolysis by hemicellulases, the one or more hemicellulases in the composition of the first aspect can be present in an amount of between about 0.1 mg/g and about 3.0 mg/g, or about 0.2 mg/g to about 2.5 mg/g, or about 0.5 mg/g to about 2.0 mg/g, or about 0.7 mg/g to about 2.5 mg/g, or about 0.2 mg/g to about 1.5 mg/g, or even about 0.2 mg/g to about 2.0 mg/g, of dry solids.

[23] In certain embodiments, the starch in the composition of the first aspect is obtained from corn, wheat, barley, sorghum, rye, sweet sorghum, rice, rye, potatoes, or other suitable grains, or any combinations thereof. In certain preferred embodiments, the starch is from corn.

[24] In some embodiments, the composition of the first aspect further comprises one or more starch-degrading enzymes selected from a de-branching enzyme, a pectinase, a beta-amylase, an alpha-amylase, or a phytase. In certain embodiments, at least two among the listed starch-degrading enzymes are present, for example, an alpha amylase, and a phytase; or a pectinase and an alpha amylase; or a beta-amylase and an alpha-amylase, or a de-branching enzyme and a pectinase; or a phytase and a de-branching enzyme. In certain embodiments, at least three starch-degrading enzymes are present, for example, an alpha amylase, a phytase and a pectinase; an alpha amylase, a beta-amylase, and a phytase; or an alpha amylase, a pectinase and a de-branching enzyme, etc. In certain embodiments, the starch degrading enzymes dosed in addition to the glucoamylase in the composition of the first aspect is about 1 AAU/g DS to about 6 AAU/g DS for alpha amylase (e.g., about 1.5 AAU/g DS to about 5 AAU/g DS, about 2 AAU g/DS to about 4 AAU/g DS), and the dosing of alpha amylase can be achieved by a single addition or multiple additions of enzymes to the composition.

[25] In certain embodiments, the amount of glucoamylase used in the composition is at least about 0.2 mg/g DS, but more preferably at least about 0.3 mg/g DS, or most preferably at a level that is 0.4 mg/g DS or higher.

[26] In some embodiments, the composition of the first aspect further comprises one or more enzymes that are cellulases. For example, the cellulases may be selected from one or more of cellobiohydrolases, endoglucanases, and/or beta-glucosidases. In certain embodiments, the composition of the first aspect comprises at least two different cellulases, for example, an endoglucanase and a cellobiohydrolate; or a cellobiohydrolase and a different cellobiohydrolase; an endoglucanase and a different endoglucanase; an endoglucanase and a beta-glucosidase; or a cellobiohydrolase and a beta-glucosidase. In certain embodiments, the composition of the first aspect comprises three or more different cellulases. For example, the composition may comprise an endoglucanase and two different cellobiohydrolases; or an endoglucanase, a cellobiohydrolase and a beta-glucosidase; or two different endoglucanases and one cellobiohydrolase, etc.

[27] The amount of cellulase used in a commercial setting would often depend upon the balance of the cost associated with making, using, or purchasing the cellulases for use in the grain ethanol plants vs. the benefits associated with the small amounts of increased yield of ethanol. In certain embodiments, the amount of cellulases used in the composition is at least about 0.02 mg/g, at least about 0.05 mg/g, at least about 0.10 mg/g, at least about 0.2 mg/g, at least about 0.3 mg/g, at least about 0.35 mg/g, but more preferably at least about 0.35 mg/g DS, but more preferably at least about 0.40 mg/g DS, or about 0.45 mg/g DS, or even about 0.50 mg/g DS. For example, the amount of cellulases used in the composition of the first aspect may be as low as 0.02 mg/g, 0.03 mg/g, 0.04 mg/g, or 0.05 mg/g DS, or as high as 0.55 mg/g DS, 0.6 mg/g DS, 0.7 mg/g DS, 0.8 mg/g DS, 0.9 mg/g DS, 1.0 mg/g DS, 1.5 mg/g DS, 2.0 mg/g DS, 2.5 mg/g DS, or even higher.

[28] The amount of hemicellulases used, on the other hand, depends on quite a different set of other factors from those related to the use of cellulases, as provided herein. In certain embodiments, the one or more hemicellulases of the composition of the first aspect may be selected from a xylanase, a beta-xylosidase, and an L-alpha-arabinofuranosidase. In some embodiments, the composition of the first aspect comprises at least two hemicellulases, for example, a xylanase, and a beta-xylosidase; two different xylanases, a xylanase and an arabinofuranosidase; a beta-xylosidase and an L-alpha-arabinofuranosidase, etc. In some embodiments, the composition of the first aspect may comprise three or more hemicellulases. For example, the composition of the first aspect may comprise a xylanase, a beta-xylosidase and an L-alpha-arabinofuranosidase.

[29] In some embodiments, the amount of hemicellulases used in the composition is at least about 0.1 mg/g DS, but more preferably at least about 0.2 mg/g DS, or about 0.5 mg/g DS, or even about 0.7 mg/g DS, or even higher. In some embodiments, the amount of hemicellulases present can be expressed using more real-life, industrially-relevant units, for example, in an amount of at least about 0.1 kg/metric ton, at least about 0.2 kg/metric ton, or at least about 0.5 kg/metric ton, or even about 0.7 kg/metric ton of dry solids, or even higher. Taking into account of the theoretical content of hemicellulose in a given grain-based substrate available for hydrolysis, however, the amount of hemicellulases present in the composition can be, in certain embodiments, about 0.1 mg/g DS to about 2.5 mg/g DS, or about 0.2 mg/g DS to about 2.0 mg/g DS, or about 0.5 mg/g DS to about 1.5 mg/g DS, or about 0.2 mg/g DS to about 1.8 mg/g DS, or even about 0.7 mg/g DS to about 1.7 mg/g DS. Expressed in an industrially relevant scale, the hemicellulases are dosed at about 0.1 kg/metric ton to about 2.5 kg/metric ton of dry solids, or about 0.2 kg/metric ton to about 2.0 kg/metric ton, or about 0.5 kg/metric ton to about 1.5 kg/metric ton, or even about 0.2 kg/metric ton to about 1.8 kg/metric ton, or even about 0.7 kg/metric ton to about 1.7 kg/metric ton of dry solids.

[30] In a second aspect, a method of preparing the composition of the first aspect is provided, as well as a method of fermenting the composition with a suitable ethanologen to produce ethanol. In some embodiments, such a method results in a relatively higher yield of ethanol after fermentation and/or a relatively higher yield of soluble fermentable sugars before fermentation, as compared to a comparable process without the presence of hemicellulases. In some embodiments, the relatively higher yield is by at least about 0.1%, or about 0.2%, or about 0.3%, for example, at least about 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, or even 1%.

[31] Provided are also various ethanologens that are capable of metabolizing the soluble fermentable sugars such as glucose and/or xylose, such that the sugars can then be converted to ethanol. For example, such suitable ethanologen can be a bacterium or a fungus, such as a *Zymomonas mobilis* bacterium or a yeast, or one that is capable of fermenting the sugars under anaerobic conditions such as a *Clostridium thermocellum*. In some embodiments, the ethanologen can be one that has been engineered or genetically manipulated to have more effective and/or efficient metabolism, and as such ethanol conversion of C6 sugars. Alternatively the ethanologen can be one that has genetically manipulated by pathway engineering to introduce an added capability to metabolize, ferment and convert to ethanol also C5 sugars such as xylose and/or even arabinose in addition to the traditional C6 glucose sugars.

[32] In some respects the method of preparing the composition is preceded by the making of a liquefied starch slurry, which can comprise a starch-containing substrate such as ground corn,

incubated or jet-cooked with a enzyme solution comprising an alpha amylase at a temperature of at least 100 °C, for example, at about 102 °C, at about 105 °C, or at an even higher temperature.

[33] In certain embodiments, the liquefied starch slurry is then mixed with an enzyme composition comprising a glucoamylase in a saccharification mixture, which may optionally contains some level of alpha amylase. The enzyme composition comprising the glucoamylase, and optionally some alpha amylase further comprises hemicellulases. The mixture of the liquefied starch slurry and the enzyme composition is then incubated at a temperature of about 25 °C to about 50 °C, for example, about 30 °C to about 45 °C, about 32 °C to about 42 °C, or about 34 °C to about 40 °C, for a duration of about 6 hours to about 180 hours, for example, about 12 hours to about 165 hours, about 24 hours to about 180 hours, about 36 hours to about 165 hours, about 60 hours to about 120 hours, about 72 hours to about 180 hours, etc, to allow the enzymatic reaction to occur, thereby creating a fermentation feedstock.

[34] The enzyme composition comprising the glucoamylase may, in certain embodiments, further comprises one or more cellulases. In yet a further embodiment, the enzyme composition comprising the glucoamylase may comprises one or more cellulases and one or more hemicellulases.

[35] The one or more cellulases may be selected from endoglucanases, cellobiohydrolases, or beta-glucosidases. The one or more hemicellulases may be selected from xylanases, beta-xylosidases, or L-alpha-arabinofuranosidases.

[36] In a different aspect, a method of saccharifying a starch-containing substrate to prepare for a fermentation feedstock is provided, comprising the steps of (1) contacting a liquefied starch slurry that contains at least some cellulose and hemicellulose materials, with an enzyme mixture composition comprising a glucoamylase and one or more hemicellulases to form a substrate-enzyme mixture; and (2) incubating the substrate-enzyme mixture for a period of time, under an incubation condition suitable for enzyme activity, to allow enzymatic reactions to occur.

[37] In some embodiments, the period of incubation time for such a method is one that is sufficient to allow (1) an increased concentration of at least one fermentable sugar, including, without limitation, glucose, xylose, and other soluble small (dimer, trimer, or tetramer) polymeric sugars in the fermentation feedstock; (2) release at least one starch chain bound to or trapped by the lignocellulosic material; or (3) to hydrolyze some portion of the hemicellulosic material; as compared to when the level of (1), (2) or (3) is measured and compared to a control liquefied starch slurry not contacted with the one or more hemicelullases. In some embodiments, the period of incubation time is between 6 hours and 180 hours, for example, between 12 hours and 165 hours,

between 24 hours and 180 hours, between 24 hours and 165 hours, between 48 hours and 165 hours, between 60 hours and 180 hours, between 72 hours and 180 hours, etc.

[38] In some embodiments, the substrate-enzyme mixture of the method further comprises one or more starch-degrading enzymes selected from an alpha-amylase, a beta-amylase, a de-branching enzyme, a pectinase or a phytase. In certain embodiments, the substrate enzyme mixture may comprise at least two of the starch-degrading enzymes, for example, an alpha amylase and a pectinase; an alpha-amylase and a phytase; an alpha-amylase and a beta-amylase; an alpha amylase and a de-branching enzyme. In further embodiments, the substrate-enzyme mixture may comprise at least three of the starch-degrading enzymes, for example, an alpha-amylase, a pectinase and a phytase.

[39] In some embodiments, the substrate-enzyme mixture of the method further comprises one or more cellulases selected from endoglucanases, cellobiohydrolases, or beta-glucosidases. For example, the substrate-enzyme mixture may comprise at least two different cellulases, such as an endoglucanase and a cellobiohydrolase; a cellobiohydrolase and a beta-glucosidase; two different cellobiohydrolases; two different endoglucanases, an endoglucanase and a beta-glucosidase, etc. In certain embodiments, the substrate-enzyme mixture may comprise at least three different cellulases, such as an endoglucanase and two different cellobiohydrolases; an endoglucanase, a cellobiohydrolase, and a beta-glucosidase tec.

[40] In certain embodiments, the one or more hemicellulases of the substrate-enzyme mixture may be selected from xylanases, beta-xylosidases, or L-alpha-arabinofuranosidases. For example, the substrate-enzyme mixture may comprise at least two different hemicellulases, such as a xylanase and a beta-xylosidase; a xylanase and an L-alpha-arabinofuranosidase; a beta-xylosidase and an L-alpha-arabinofuranosidase; two different xylanases, two different beta-xylosidases, etc. In certain embodiments, the substrate-enzyme mixture may comprise at least three different hemicelulases, such as, for example, two different xylanases and a beta-xylosidase; two different xylanases and an L-alpha-arabinofuranosidase; a xylanase, a beta-xylosidase and an L-alpha-arabinofuranosidase. In some embodiments, the hemicellulases in the enzyme-substrate mixture is about 0.1 kg/metric ton to about 2.5 kg/metric ton, for xample, about 0.2 kg/metric ton to about 2.0 kg/metric ton, about 0.3 kg/metric ton to about 2.2 kg/metric ton, about 0.5 kg/metric ton to about 1.9 kg/metric ton, about 0.7 kg/metric ton to about 2.2 kg/metric ton of dry solid.

[41] In certain embodiments, the conditions of incubation suitable for enzyme activity to be used with the method of the third respect comprises a pH of about 3.5 to about 8.5, for example, about 3.5 to about 8, about 4 to about 8, about 4 to about 7.5, about 4.5 to about 8.5, about 4.5 to about 8, or even about 4 to about 7; a temperature of about 25 °C to about 50 °C, for example, about 25 °C

to about 45 °C, about 25 °C to about 40 °C, about 30 °C to about 50 °C, about 30 °C to about 45 °C, about 30 °C to about 45 °C, or even about 35 °C to about 45 °C; and an agitation rate of about 50 to about 200 RPM, for example, about 70 to about 150 RPM, about 80 to about 175 RPM, about 90 to about 120 RPM, or even about 86 to about 110 RPM.

[42] In certain embodiments, the method of this aspect further comprises fermenting the fermentation feedstock to produce a fermentation product. In some embodiments, the fermentation product is an ethanol. In further embodiments, the yield of fermentation product, for example, ethanol, is higher by at least about 0.1% to about 5%, as compared to the yield of the same fermentation product from a comparable process without the presence of the one or more hemicellulases in the substrate-enzyme mixture.

[43] In certain embodiments, the total time for incubation and fermentation steps ranges from 6 hours to 180 hours, for example, from 12 hours to 165 hours, or from 24 hours to 180 hours, or even from 48 hours to 165 hours. In certain particular embodiments, the incubation step or at least a part of the incubation step and fermentation step are conducted simultaneously in the same vessel.

[44] In some embodiments of all of the aspects of the invention, the starch is obtained from plant or other suitable sources such as, for example, corn, wheat, barley, sorghum, rye, sweet sorghum, rice, rye, potatoes, or other suitable grains. In certain particular embodiments, the starch is a mixture of starch from different plant sources. In some embodiments, the starch is obtained from corn.

[45] In certain other embodiments, the incubation and the fermentation steps are separate and distinct. In particular instances, there is an added pretreatment step of the incubated mixture or a portion thereof (e.g., the liquid phase, the solid phase, or a mixture of both phases) between the incubation step and the fermentation step.

[46] These and other aspects and embodiments of present methods and variant cells will be apparent from the description, including the appended Examples and accompanying Figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[47] **Figure 1** is a simplified depiction of a typical or traditional dry grind grain ethanol production process.

[48] **Figures 2A-2O** depict the amounts of glucose, xylose, glycerol and ethanol produced from the experiment of Example 1. The aliquoted samples of SSF broths were taken at various time points throughout the SSF process, and each component was measured. The product

compositions of the 4 parallel SSF processes of Example 1 were determined using HPLC, which traces are depicted in **Figure 2D-2O.**

DETAILED DESCRIPTION

I. Overview

[49] Described is a method of improving the production of ethanol from grain-based plant material feedstock such as corn. More specifically, the improved method is one that involves the addition of an enzyme mixture comprising at least a certain threshold level of hemicellulases, optionally in addition to cellulases and other auxiliary enzymes to a saccharification mixture, together with a glucoamylase and/or an amylase. Optionally and in a key embodiment, the saccharification mixture is one and the same as the fermentation mixture as used in a simultaneous saccharification and fermentation (SSF) step.

[50] The improved process gives yields of higher ethanol per bushel of corn, as compared to a similar utilizing less (below the threshold level of) such hemicellulases or no hemicellulase at all in the saccharification step. When hemicelluases are added to the saccharification step, but at a level below the threshold level, the substantial pressure buildup downstream, for example, in the post-distillation mixture and thin stillage, gives rise to the real risk of overall process failure and a complete shutdown of the production run.

[51] At below the threshold level of hemicellulases, the pressure buildup downstream is faster or more immediate as the amount of hemicellulases increases. It is thought that the downstream pressure buildup is caused by the attendant significant rise in viscosity and/or thickness in of the post distillation mixture and the thin stillage. The high viscosity and thickness of the thin stillage makes it much more difficult to evaporate effectively. While this vicsocity buildup may be relieved by adding significant amount of fresh water to the thin stillage, to, for example, make it more pumpable, such a dilute thin stillage at a much greater volume becomes much more difficult to evaporate without cripplingly high expenditures of water and energy, which would typically still result in a diluted syrup with greatly reduced nutritional value than one that is produced in a conventional dry grind ethanol plant. It is also completely counterproductive in the sense that the whole point of evaporating the thin stillage is to reduce the amount of water therein, potentially recycling at least a part of that process water as backset therefore reducing the process water consumption.

[52] It is believed that the imcompletely broken down arabinoxylan oligomers, which have greater affinity for water so much so as to sequester water, at a lower level of hemicellulase

dosing, reduce the effectiveness of evaporation, makes thin stillage less pumpable, and incurs significantly increased energy expenditures.

[53] Typically a portion of the water recovered from the evaporator (from evaporating the thin stillage) is re-cycled or re-used as part of the liquid added to the plant feedstock (pre-reduced through grinding, for example) to form the initial slurry on which the saccharification enzymes are added to effect the saccharification process. In many instances, the water added to the reduced feedstock to form slurry can be about 1 vol% up to about 70 vol% water recovered or reused from the stillage, such as about 15 vol% to about 60 vol%, or about 30 vol% to about 50 vol%, or about 40 vol% stillage. The inability to evaporate from the thin stillage caused by the difficulties of pumping a material with significantly increased viscosity and thickness also leads to less amount of water (backset) being made available to be recycled. As such, fresh water needs to be added to the pre-reduced grain feedstock in order to arrive at a suitable initial slurry for the initial liquefaction step. This further upsets the water usage balance of the grain ethanol plant.

[54] It is therefore impractical and unjustifiable to use water addition to remedy the pressure, in view of the dramatically increased energy and water expenditures, and the reduced quality and usefulness of the co-product.

[55] However, it has been surprisingly discovered that, the true and commercially advantageous remedy to the pressure buildup issues in this case is not to reduce the amount of hemicellulases added to the saccharification step. On the contrary, the amount of hemicellulases should be significantly increased to a level above a certain threshold whereby not only is a higher ethanol yield achievable (potentially from the trapped residual starch as well as the broken down soluble hemicellulosic sugars from the hemicellulose components of the grain), the downstream viscosity issues are resolved. As such the solids/liquid separation of the post-distillation mixture as well as the evaporation and condensation of thin stillage can carry on as in a conventional grain ethanol plant, the amount of backset produced remains sufficient to be used in the making of the initial slurry for saccharification in the next run, and most importantly, an increased overall yield of ethanol produced from the process is increased.

[56] It is known that the fibrous materials of the grain-based plant biomass contain both cellulose and hemicelluloses. The addition of cellulases to the saccharification mixtures can help to break down the cellulose part of the fibrous materials, thereby releasing some of the bound or entrapped starch that would otherwise not be processed by the saccharification enzymes. On the other hand, it is also known that hemicellulases and cellulases, when combined in a single mixture in the presence of a lignocellulosic biomass material comprising both

cellulose and hemicelluloses, act synergistically to break down cellulose, resulting in a higher glucan conversion than would typically occur in the presence of the same amount of cellulase without the presence of the hemicellulases. Furthermore, it is known that hemicellulases, even in the presence of cellulases, often cannot break down the hemicelluloses completely, manifested in a xylan conversion level that can be lower than the glucan conversion levels, although that may well be due to the differing efficacies of particular hemicellulases and cellulases in a given enzyme mixture.

[57] It is believed that addition of hemicellulases indeed helps to break down the hemicellulose part of grain feedstocks itself as well as confers synergies to any cellulase activities that may also be present in the saccharification step. However, at a level under the threshold as described herein, the hemicellulose breakdown will be incomplete, resulting in the production of significant amount of arabinoxylan oligomers that not only causes the increase in thickness and/or viscosity in the thin stillage due to the insoluble breakdown products of arabinoxylan oligomers, but also makes the thus-created thin stillage difficult to evaporate because the incompletely broken-down arabinoxylan oligomers sequesters water.

[58] As the level of hemicellases increases to above a threshold in the saccharification mixture, a more complete degradation of the hemicelluloses is achieved, giving yield to a post-distillation mixture comprising sufficiently high levels of soluble monomeric sugars rather than insoluble incompletely degraded arabinoxylan oligomers, and a normal thickness/viscosity in the thin stillage. The thin stillage resulting from the high hemicellulase saccharification can be evaporated normally, giving rise to a syrup of normal nutritional value, with potentially a slight variation of such nutritional value related to the inclusion of xylose and arabinose monomeric sugars in the syrup, and sufficient and normal level of backset that can then be recycled/reused to prepare the slurry for the next production run.

II. Definitions

[59] Before the present compositions and methods are described in greater detail, it is to be understood that the present compositions and methods are not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present compositions and methods will be limited only by the appended claims.

[60] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the

upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the present compositions and methods. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the present compositions and methods, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the present compositions and methods.

[61] Certain ranges are presented herein with numerical values being preceded by the term "about." The term "about" is used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number may be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number. For example, in connection with a numerical value, the term “about” refers to a range of -10% to +10% of the numerical value, unless the term is otherwise specifically defined in context. In another example, the phrase a “pH value of about 6” refers to pH values of from 5.4 to 6.6, unless the pH value is specifically defined otherwise.

[62] The headings provided herein are not limitations of the various aspects or embodiments of the present compositions and methods which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

[63] The present document is organized into a number of sections for ease of reading; however, the reader will appreciate that statements made in one section may apply to other sections. In this manner, the headings used for different sections of the disclosure should not be construed as limiting.

[64] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present compositions and methods belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present compositions and methods, representative illustrative methods and materials are now described.

[65] All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated

to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present compositions and methods are not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[66] In accordance with this detailed description, the following abbreviations and definitions apply. Note that the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an enzyme" includes a plurality of such enzymes, and reference to "the dosage" includes reference to one or more dosages and equivalents thereof known to those skilled in the art, and so forth.

[67] It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[68] It is further noted that the term "consisting essentially of," as used herein refers to a composition wherein the component(s) after the term is in the presence of other known component(s) in a total amount that is less than 30% by weight of the total composition and do not contribute to or interferes with the actions or activities of the component(s).

[69] It is further noted that the term "comprising," as used herein, means including, but not limited to, the component(s) after the term "comprising." The component(s) after the term "comprising" are required or mandatory, but the composition comprising the component(s) may further include other non-mandatory or optional component(s).

[70] It is also noted that the term "consisting of," as used herein, means including, and limited to, the component(s) after the term "consisting of." The component(s) after the term "consisting of" are therefore required or mandatory, and no other component(s) are present in the composition.

[71] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present compositions and

methods described herein. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

[72] Enzymes have traditionally been classified by substrate specificity and reaction products. In the pre-genomic era, function was regarded as the most amenable (and perhaps most useful) basis for comparing enzymes and assays for various enzymatic activities have been well-developed for many years, resulting in the familiar EC classification scheme. Cellulases and other glycosyl hydrolases, which act upon glycosidic bonds between two carbohydrate moieties (or a carbohydrate and non-carbohydrate moiety--as occurs in nitrophenol-glycoside derivatives) are, under this classification scheme, designated as EC 3.2.1.-, with the final number indicating the exact type of bond cleaved. For example, according to this scheme an endo-acting cellulase (1,4-β-endoglucanase) is designated EC 3.2.1.4.

[73] With the advent of widespread genome sequencing projects, sequencing data have facilitated analyses and comparison of related genes and proteins. Additionally, a growing number of enzymes capable of acting on carbohydrate moieties (i.e., carbohydrases) have been crystallized and their 3-D structures solved. Such analyses have identified discreet families of enzymes with related sequence, which contain conserved three-dimensional folds that can be predicted based on their amino acid sequence. Further, it has been shown that enzymes with the same or similar three-dimensional folds exhibit the same or similar stereospecificity of hydrolysis, even when catalyzing different reactions (Henrissat *et al.*, FEBS Lett 1998, 425(2): 352-4; Coutinho and Henrissat, Genetics, biochemistry and ecology of cellulose degradation, 1999, T. Kimura. Tokyo, Uni Publishers Co: 15-23.).

[74] These findings form the basis of a sequence-based classification of carbohydrase modules, which is available in the form of an internet database, the Carbohydrate-Active enZYme server (CAZy), available at http://afmb.cnrs-mrs.fr/CAZY/index.html (Carbohydrate-active enzymes: an integrated database approach. See Cantarel *et al.*, 2009, Nucleic Acids Res. 37 (Database issue):D233-38).

[75] CAZy defines four major classes of carbohydrases distinguishable by the type of reaction catalyzed: Glycosyl Hydrolases (GH's), Glycosyltransferases (GT's), Polysaccharide Lyases (PL's), and Carbohydrate Esterases (CE's). The enzymes of the disclosure are glycosyl hydrolases. GH's are a group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. A classification system for glycosyl hydrolases, grouped by sequence similarity, has led to the definition of over 85 different families. This classification is available on the CAZy web site.

[76] The term “protein", as used herein, includes proteins, polypeptides, and peptides.

[77] As used herein, the term “amino acid sequence” is synonymous with the term “polypeptide” and/or the term “protein”. In some instances, the term “amino acid sequence” is synonymous with the term “peptide”. In some instances, the term “amino acid sequence” is synonymous with the term “enzyme”.

[78] The terms "protein" and "polypeptide" are used interchangeably herein. In the present disclosure and claims, the conventional one-letter and three-letter codes for amino acid residues may be used. The 3-letter code for amino acids as defined in conformity with the IUPAC IUB Joint Commission on Biochemical Nomenclature (JCBN). It is also understood that a polypeptide may be coded for by more than one nucleotide sequence due to the degeneracy of the genetic code.

[79] “Amylase” means an enzyme that is, among other things, capable of catalyzing the degradation of starch, amylose, amylopectin, and the like. Generally, amylases include (a) endo-cleaving enzyme activity (e.g. as found in α-amylases (EC 3.2.1.1; α-D-(1→4)-glucan glucanohydrolase)) cleaving α-D-(1→4) O-glycosidic linkages in a polysaccharide containing three or more α-D-(1→4) linked glucose units, and (b) the exo-cleaving amylolytic activity that sequentially cleaves the substrate molecule from the non-reducing end. Examples of the latter are found in β-amylases (EC 3.2.1.2), which produce β-maltose. β-Amylases, α-glucosidases (EC 3.2.1.20; α-D-glucoside glucohydrolase), glucoamylase (EC 3.2.1.3; α-D-(1→4)-glucan glucohydrolase), and product-specific amylases can produce malto-oligosaccharides of a specific length from their respective substrates.

[80] “Alpha-amylase” (e.g., E.C. 3.2.1.1) generally refers to enzymes that catalyze the hydrolysis of alpha-1,4-glucosidic linkages. These enzymes effect the hydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing 1,4-α-linked D-glucose units. The alpha-amylases release the reducing groups in the α-configuration. For the purpose of the present disclosure, “alpha-amylase” particularly includes those alpha amylase enzymes having relatively high thermostability, i.e., with sustained activity at high temperatures. For example, alpha-amylases are useful for liquefying starch at temperatures above 80°C.

[81] “Glucoamylases” are a type of exo-acting amylase that release glucosyl residues from the non-reducing ends of amylose and amylopectin molecules. Glucoamylases also catalyze the hydrolysis of α-1,6 and α-1,3 linkages, although at much slower rate than α-1,4 linkages. Glucoamylase activity can be expressed in “glucoamylase units” (GAU).

[82] As used herein, “cellulases” refer to all enzymes that hydrolyzes cellulose, i.e., any of its components, e.g., 1,4-beta-D-glycosidic linkages in cellulosic materials such as those found in

various plants and plant-related or –derived materials, such as grains, seeds, cereals, etc., or plant cell walls.

[83] As typically known, "cellulase" comprises at least the enzymes classified in E.C. 3.2.1.4 (cellulase/endocellulases or endoglucanases), E.C. 3.2.1.91 (exocellulases), and E.C. 3.2.1.21 (cellobiases or beta-glucosidases). Examples of endocellulases include endo-1,4-beta-glucanase, carboxymethyl cellulase (CMCase), endo-1,4-beta-D-glucanase, beta-1,4-glucanase, beta-1,4-endoglucan hydrolase, celludextrinase, and various endoglucanases such as those produced by naturally-occurring wood-rotting fungi. Examples of exocellulases include cellobiohydrolases, which in turn includes those that cleave the 1,4-beta-D-glycosidic linkages from the reducing ends of the cellulose chain and those that cleaves the same linkages from the non-reducing ends.

[84] "Cellulases" may also refer to complete enzyme systems that are useful for efficiently converting crystalline cellulose to glucose. Such complete cellulase system typically would comprise components from each of the cellobiohydrolase, endoglucanase and beta-glucosidase classifications, as it has been reported that individual isolated components are less effective in hydrolyzing crystalline cellulose (Filho *et al.,* Can. J. Microbiol., 42:1-5, 1996).

[85] A synergistic relationship has been observed between cellulase components from different classifications. Endo-1,4-beta-glucanases (EG) and exo-cellobiohydrolases (CBH) catalyze the hydrolysis of cellulose to cellooligosaccharides (cellobiose as a main product), while beta-glucosidases (BGL) convert the oligosaccharides to glucose. In particular, the EG-type cellulases and CBH-type cellulases synergistically interact to efficiently degrade cellulose. The beta-glucosidases serve the important role of liberating glucose from the cello-oligosaccharides such as cellobiose, which is toxic to the microorganisms that are used to ferment the sugars into ethanol (e.g., yeasts) and which is also inhibitory to the activities of endoglucanases and cellobiohydrolases, thus rendering them ineffective in further hydrolyzing the crystalline cellulose.

[86] "Cellulases" may further refer to complete enzyme systems that comprises not only cellulases but also certain hemicellulases, or any combination thereof.

[87] A number of commercial cellulase compositions are available and suitable for use in the methods and compositions described herein, including, for example, products of Genencor, Danisco US Inc., such as ACCELLERASE® 1000 and ACCELLERASE® 1500, ACCELLERASE® BG, ACCELLERASE® DUET, and ACCELLERASE® TRIO™; products of Novozymes, such as its Celluclast, Novozyme 188, Cellic CTec2, Cellic CTec3; products of AB Enzymes, such as its Flashzyme; products of Codexis, such as its CodeXyme® cellulase products; products of Dyadic, such as its CMax® products. Certain of the commercial

compositions as listed above also contains hemicellulases. For example, about 1/5 to 1/4 of the total proteins of ACCELLERASE® DUET are hemicellulases, and about 1/3 of the proteins in ACCELLERASE® TRIO™ are hemicellulases. CMax®, certain of CodeXyme® products, as well as Cellic Ctec3 all contain certain amounts of hemicellulases.

[88] The term "endoglucanase" as used herein refers to an enzyme of classification E.C. 3.2.1.4, which catalyzes the hydrolysis of 1,4-beta-D-glycosidic linkages that are found in cellulosic materials. Methods of measuring endoglucanase activities are known, including, for example, the one measuring the hydrolysis of carboxymethyl cellulose (CMC) as described by Ghose, 1987, Pure & App. Chem, 59:257-268.

[89] The term "cellobiohydrolase" refers to an enzyme with cellobiohydrolase activity or capable of catalyzing the hydrolysis of a particular glocosidic linkage in cellulose. Specifically, the cellobiohydrolase (CBH) activity may be CBH class I (CBH I) or CBH class II (CBH II) activity or a combination of both CBH I and CBH II. Suitably the cellobiohydrolase may hydrolyse (1→4)-β-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains. Another term for cellobiohydrolase activity may be exo-cellobiohydrolase activity or cellulose 1,4 β-cellobiosidase activity. The cellobiohydrolase II activity can be classified under E.C. classification EC. 3.2.1.91. The cellobiohydrolase I activity can be classified under E.C. classification EC. 3.2.1.176.

[90] The term "beta-glucosidase" as used herein refers to an enzyme having beta-glucosidase activity or one that is capable of catalyzing the hydrolysis of terminal non-reducing β-D-glucosyl residues and release of monomer β-D-glucose from cellobiose. β-glucosidase activity can be classified under E.C. classification E.C. 3.2.1.21.

[91] The term "hemicellulase" as used herein refers to a group of enzymes capable of catalyzing the hydrolysis of a hemicellulosic materials. The term "hemicellulases" as used herein refer to three major types of enzymes: beta-xylosidases, L-α-arabinofuranosidases, and xylanases. Those enzymes include, for example, arabinases, arabinofuranosidases, certain acetylmannan esterases, acetylxylan esterases, feruloyl esterases, mannanases, mannosidases, xylanases, and xylosidases, etc. Hemicellulases can be from many different glycosyl hydrolase families, including, without limitation, beta-xylosidases of GH3; beta-xylosidases of GH39; L-α-arabinofuranosidase (EC 3.2.1.55), β-xylosidase (EC 3.2.1.37), endo-arabinanase (EC 3.2.1.99), and/or galactan 1,3-β-galactosidase (EC 3.2.1.145) of GH43; and L-α-arabinofuranosidase (EC 3.2.1.55) of GH51, as well as the xylanases of GH10 and GH11, and the beta-xylosidases of GH30, for example.

[92] The term "xylanase" refers to a 1,4-beta-D-xylan xylohydrolase of E.C. 3.2.1.8, which catalyzes the hydrolysis of 1,4-beta-D-xylosidic linkages in xylan. Xylanase activities can be measured, for example, by the PHBAH assay as described by Lever, 1972, A new reaction for colorimetric determination of carbohydrates, Anal. Biochem. 47:273-279.

[93] β-xylosidase activity may hydrolyse successive xylose residues from the non-reducing termini of (1→3)-β-D-xylans, e.g. the β-xylosidase may be a 1,3 β-D-xylosidase. 1,3 β-D-xylosidases may be classified under E.C. classification E.C. 3.2.1.72 or may catalyse the hydrolysis of (1→4)-β-D-xylans, to remove successive D-xylose residues form the non-reducing termini, e.g. the β-xylosidase may be a 1,4 β-xylosidase. 1,4 β-xylosidases may be classified under E.C. classification E.C. 3.2.1.37.

[94] L-alpha arabinofuranosidases may hydrolyse (1→6)-β-D-galactosidic linkages in arabinogalactan proteins and (1→3):(1→6)-β-galactans to yield galactose and (1→6)-β-galactobiose. L-alpha-arabinofuranosidases may be classified under E.C. classification E.C. 3.2.1.164.

[95] The term "saccharification enzyme" refers to an enzyme that can catalyze conversion of a component of biomass to fermentable sugars. In the context of cellulosic conversion, it is often the case that the enzyme is more effective at producing fermentable sugars when the biomass is pretreated. However, for purposes herein the plant biomass associated with grains that are used in the grain-to-ethanol process can be subject to certain pretreatment methods that are well known in the bio-sourced ethanol industry, but it is potentially unnecessary because the cellulosic and hemicellulosic components of grains can be more accessible to enzymes than the same components in plant materials such as stalks, stems, leaves, braches, cobs and stovers.

[96] The term "microorganism" as used herein refers to any bacterium, yeast, or fungal species.

[97] As used herein the term "ethanologen" and "ethanologenic microorganism" are used interechangeably to refer to a microorganism with the ability to conert a sugar or oligosaccharide to ethanol. The etnologenic microorganism are ethanologenic by virtue of their ability to express one or more enzymes that individually or collectively convert soluble sugars to ethanol.

[98] Such an ethanolgen can also be referred as an "ethanol producing microorganism" which is an organism or cell that is capable of producing ethanol from a hexose or a pentose. Generally ethanol producing cells would contain at least one alcohol dehydrognase and a pyruvate decarboxylase. Examples of ethanol producing microorganisms include fungal microorganisms such as yeast, such as, for example, the species and strains of *Saccharomyces, e.g., S. cerevisiae.*

[99] The term "heterologous" with reference to a polynucleotide or polypepide/protein refers to a polynucleotide or polypeptide/protein that does not naturally occur in a host cell. In some embodiments, the protein is a commercially important industrial protein. It is intended that the term encompass proteins that are encoded by naturally occurring genes, mutated genes, and/or synthetic genes.

[100] The term "endogenous" as used herein with reference to a polynucleotide or polypeptide/protein refers to a polynucleotide or polypeptide/protein that occurs naturally in the host cell.

[101] As used herein, the term "starch" refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylase and amylopectin with the formula $(C_6H_{10}O_5)x$, wherein X can be any positive integer. In particular, the term refers to any plant-based material including but not limited to grains, grasses, tubers, and roots. Preferably the starchy material is wheat, barley, corn, rye, oats, rice, sorghum or milo, sweet sorghum, brans, cassava, millet, potato, sweet potato, and tapioca. For purposes herein "sorghum" generally includes "grain sorghum" also known as "milo."

[102] As used herein, the term "slurry" refers to an aqueous mixture containing at least some insoluble solids. A slurry can also contain one or more soluble components. For example, milled grain, flour, or starch are often suspended in a water-based solution to form a slurry for testing amylases or for liquefaction proposes.

[103] As used herein, the term "liquefaction" means a process by which starch is "liquefied" or converted to less viscous and shorter chain soluble dextrins, or by which the lignocellulosic material is broken down sufficiently such that the appearance of the material changes from a mostly solid form to a more liquid and pumpable form. The process of liquefying involves gelatinization of starch simultaneously with, or followed by the addition of at least an alpha-amylase. Thus liquefaction is the stage in which gelatinized starch is enzymatically hydrolyzed, e.g., thereby reducing the chain length of the starch and concomitantly, the viscosity. As used herein "liquefact" refers to the liquefied starch slurry, i.e., the resultant hydrolyzed mixture. Such a liquefact is generally the starting material for a saccharification process which may be conducted in conjunction, simultaneously, or in separate and distinct steps from fermentation. As used herein, the term "gelatization" or "gelatinized" means solubilization or a solublized form of a starch molecule achieved through cooking of the starch material to form a viscous suspension.

[104] As used herein, the terms "saccharification" refers to enzymatic conversion of starch and/or lignocellulosic materials, which have typically been subject to one or more pretreatment

methods, to glucose. In the context of starch saccharification, for example, after liquefaction, a starch slurry can be saccharified to convert the maltodextrins to fermentable sugars, e.g., glucose, maltose, etc. Saccharification typically involves the use of enzymes, particularly glucoamylases but also debranching enzymes and other suitable enzymes in the context of starch saccharification to glucose. Saccharification in the context of lignocellulosic biomass materials also involves the use of suites of enzymes or enzyme cocktails, particularly various cellulases, such as for example, cellobiohydrolases, endoglucanases, and beta-glucosidases.

[105] The term "the degree of polymerization (DP)" refers to the number (n) of anhydroglucopyraonse units in a given saccharide. Examples of DP1 are the monosaccharides, such as glucose and fructose. Eamples of DP2 are the disaccharides, such as maltose and sucrose. A DP>3 refers to polymers with a degree of polymerization of greater than 3. DP can be used as a parameter to measure relative degree of breakdown of starch (high DP) to sugars (low DP). The same term can also be used in the context of lignocellulosic biomass breakdown products, whereas, for example, monosaccharides like glucose and xylose can be referred to as DP1 sugars, disaccharides like cellobiose can be referred to as DP2 sugars, etc.

[106] The term "simultaneous saccharification and fermentation (SSF)" refers to a process wherein a step of scarifying a raw material (e.g., a whole grain, a liquefact , or other biomass comprising starch and/or cellulosic materials) and a fermentation step are combined into a single process that is conducted together in the same vessel. SSF can, in some embodiment, be conducted in conjunction with process steps that are saccharification alone or fermentation alone, for example, in a process that starts with saccharification alone with enzymes in a vessel for a period, followed by the addition of an ethanologen microorganism into the saccharification mixture, which has achieved a certain degree of saccharification during the saccharification step, to perform an SSF step. The SSF step in this case can optionally be followed with a fermentation alone step wherein the saccharification of the enzyme substrate is completed and wherein the fermenting organism utilizes and ferment the sugars that have been produced during the now –completed saccharification step. Alternatively, SSF can for example be the sole step in a grain to ethanol process during which either saccharification or fermentation occurs. In other words, the grain-to-ethanol process does not have any separate saccharification or fermentation steps beside the SSF step during which both saccharification and fermentation take place.

[107] "Activity" with respect to enzymes means catalytic activity and encompasses any acceptable measure of enzyme activity, such as the rate of activity, the amount of activity, or the specific activity. As used herein, "specific activity" means an enzyme unit defined as the

number of moles of substrate converted to product by an enzyme preparation per unit time under specific conditions. Specific activity is expressed as units (U)/mg of protein.

[108] "Alpha-amylase unit" (AAU) refers to alpha-amylase activity measured according to the method disclosed in U.S. Patent No. 5,958,739. In brief, the assay uses p-nitrophenyl maltoheptoside (PNP-G7) as the substrate with the non-reducing terminal sugar chemically blocked. PNP-G7 can be cleaved by an endo-amylase, for example alpha-amylase. Following the cleavage, an alpha-glucosidase and a glucoamylase digest the substrate to liberate free PNP molecules, which display a yellow color and can be measured by visible spectrophotometry at 410 nm. The rate of PNP release is proportional to alpha-amylase activity. The AAU of a given sample is calculated against a standard control. One unit of AAU refers to the amount of enzyme required to hydrolyze 10 mg of starch per minute under specified conditions.

[109] As used herein the term "cellulose" or "cellulosic materials" refers to materials containing cellulose. Relatedly, the term "lignocellulose" or "lignocellulosic materials" refers such cellulosic materials that also contain lignin. It is known that the largest component polysaccharides constituting the cell walls of plant biomass include cellulose, hemicelluloses and pectin. Cellulose is an organic compound with the formula $(C_6H_{10}O_5)n$, representing a polysaccharide consisting of a linear chain of $\beta(1\rightarrow4)$ linked D-glucose units.

[110] "Hemicellulose" refers to any of several heteropolymers (matrix polysaccharides) present along with cellulose in almost all plant cell walls, interconnecting the insoluble crystalline matrix of cellulose, which are further embedded or connected to lignin that help to provide for the physical integrity of the plants. Hemicellulases may be xylan, glucuronoxylan,xyloglucans, arabinoxylans, glucomannan, and mannans. When hemicellulose is broken down into sugar monomers, the monomers may include xylose, mannose, galactose, rhamnose, and arabinose, which are mostly D-pentose (C-5 sugars), and occasionally small amounts of L-sugars as well. Xylose is in most cases the most abundant sugar monomer, although in softwoods mannose can be the most abundant sugar. Not only regular sugars can be found in hemicellulose, but also their acidified form, for instance glucuronic acid and galacturonic acid can be present.

[111] Cellulose and lignocellulose are found in various plants and plant-derived materials, including stems, leaves and cobs, various parts of grains, including, for example, corn fiber, wheat hull, etc. Cellulosic materials or lignocellulosic materials can also be materials produced from plants and plant parts, such as paper and pulp. The term "cellulosic" refers to a composition comprising cellulose and additional components, including, for example, hemicellulose.

[112] Suitably the lignocellulosic materials of the invention is from an agricultural plant material including a grain, for example, a starch-based grain feedstock such as corn, barley, etc, from which starch-based ethanol has been produced and applied by the industry for many years.

[113] The term "simultaneous saccharification and fermentation" or "SSF" refers to a process or reaction configuration wherein biomass is saccharified and the fermentable sugars produced from the saccharification are used by an enzyme and/or by a fermenting microorganism to produce a product all at the same time, typically in the same reaction vessel.

[114] The term "hybrid saccharification and fermentation" or "HSF" refers to a process or reaction configuration wherein the plant materials, for example, the grain-based plant materials including starch and cellulosic and hemicellulosic components, are saccharified to a limited extent (incomplete or partial saccharification), followed by continued saccharification and fermentation occurring simultaneously.

[115] The terms "separate saccharification and fermentation," "separate hydrolysis and fermentation," and "SHF" are used interchangeably herein. They refer to a process or reaction configuration wherein the plant materials, for example, the grain-based plant materials including starch and cellulosic and hemicellulosic components, are saccharified or hydrolyzed to substantial completion (*e.g.,* about 60% or more complete, about 70% or more complete, about 80% or more complete, about 90% or more complete, or about 95% or more complete) or to completion (*e.g.,* about 99% or more complete, or about 100% complete, such that all fermentable sugars that would be released from a given saccharification reaction are released), followed by a separate and distinct fermentation step, wherein the fermentation sugars produced by the saccharification or hydrolysis step is fermented to produce a fermentation product.

[116] The term "fermentable sugar" refers to oligosaccharides and monosaccharides that can be used as a carbon source by a microorganism in a fermentation process.

[117] The term "partial saccharifcation" refers to limited saccharification of plant-based materials, such as, for example, grain-based materials such as starch and cellulosic or hemicellulosic materials, where the fermentable sugars released are less than the total of fermentable sugars that would be released if saccharification is run to completion.

[118] The term "saccharification" refers to the production of fermentable sugars from polysaccharides or polysaccharide-containing materials.

[119] The term "lignocellulosic materials" refers to any materials comprising ellulosic, hemicellulosic and lignin components. Suitably for the present disclosure, the lignocellulosic materials may also comprise starch, oligosaccharides, and/or monosaccharides, even proteins or lipids.

[120] The term "derived" encompasses the terms "originated from," "obtained" or "obtainable from," and "isolated from."

[121] The term "fermentation" as used herein refers to the enzymatic and/or anaerobic breakdown of organic substances by microorganisms to produce simpler organic compounds. Although fermentation occurs under anaerobic conditions, the term "fermentation" as used herein is not intended to be limited to strict anaerobic conditions, because fermentation also can occur in the presence of oxygen at various levels. Accordingly fermentation encompasses at least some fermentative conversion of a starch substrate containing granular starch to an end product in the context of a starch fermentation, and in the context of a fermentation of cellulosic sugars, the conversion of a soluble cellulosic sugars into an end product.

[122] The term "contacting" as used herein refers to placing of the enzyme(s) in a reactor, vessel or the like, such that the enzymes can come into sufficiently close proximity to the substrate so as to enable the enzymes to convert the substrate to the end product. The skilled persons in the art would recognize that mixing an enzyme (e.g., in a solution form) with one or more substrates, whether in a relatively pure or crude form, constitutes contacting.

[123] As used herein the term "dry solids (DS) content" or "dry solids (DS) level" refers to the total solids of a mixture of solids and liquids (e.g., slurry) on a dry weight basis. Dry solids content and dry weight basis are usually expressed, for example, as the weight of the subject material as a percentage of the weight of the total material to which the dry solids are a part.

[124] The term "residual starch" refers to the amount of starch present in grain by-product after fermentation. Typically the amount of residual starch present in 100 grams of DDGS may be one of the parameters to evaluate the efficiency of starch utilization in a fermentation process, such as one that is used to produce ethanol.

[125] The term "mash" refers to a mixture of fermentable carbon source (carbohydrate) in water used to produce a fermented product such an alcohol. The term "beer" and "mash" can be and are used interchangeably herein.

[126] The term "stillage" as used herein refers to a mixture of non-fermented solids and water, which is the residue after removal of alcohol from a fermented mash.

[127] Stillage coming from the distillation (e.g. comprising water, remainings of the grain, yeast cells etc.) is separated into a "solid" part and a liquid part. The solid part is called "wet-cake" and can be used as animal feed as such. The liquid part is (partially) evaporated into a syrup (solubles). When the wet-cake is dried it is Distillers Dried Grains (DDG). When the wet-cake is dried together with the syrup (solubles) it is Distillers Dried Grans with Solubles (DDGS). Wet-cake may be used in dairy operations and beef cattle feedlots. The dried DDGS

may be used in livestock, e.g. dairy, beef and swine, feeds and poultry feeds. DDGS is a good protein source for dairy cows.

[128] The term "whole stillage" as used herein means the grains and liquid effluent remaining after distillation.

[129] By-products from industrial processing of cereals and other plants, such as wet-cake, Distillers Dried Grain, Distillers Dried Grain Solubles (DDGS)), wheat bran, wheat middlings, wheat shorts, rice bran, rice hulls, oat hulls, palm kernel, and citrus pulp are normally high in fibre (cellulose and/or hemicellulose) and are not all readily digestible by animals (particularly monogastric animals) and are used in animal feed.

[130] Wet-cake, Distillers Dried Grains and Distillers Dried Grains with Solubles for example are by-products obtained after the removal of ethyl alcohol by distillation from yeast fermentation of a grain or a grain mixture by methods employed in the grain distilling industry.

[131] Stillage coming from the distillation (e.g. comprising water, remainings of the grain, yeast cells etc.) is separated into a "solid" part and a liquid part. The solid part is called "wet-cake" and can be used as animal feed as such. The liquid part is (partially) evaporated into a syrup (solubles). When the wet-cake is dried it is Distillers Dried Grains (DDG). When the wet-cake is dried together with the syrup (solubles) it is Distillers Dried Grans with Solubles (DDGS). Wet-cake may be used in dairy operations and beef cattle feedlots. The dried DDGS may be used in livestock, e.g. dairy, beef and swine, feeds and poultry feeds. DDGS is a good protein source for dairy cows.

[132] The term "distillers dried grain" (DDG) and "Distillers' dried grain with soluble (DDGS)" refers to a useful by-product of grain fermentation.

[133] The terms "recovered," "isolated," and "separated" as used herein refer to a compound, protein, cell, nucleic acid, or amino acid that is removed from at least one component with which it is naturally associated.

[134] The term "yield" with reference to the ethanol production refers to the production of a compound, e.g., ethanol, from a certain amount of a starting material, e.g., a grain-based feedstock. "Yield" may be expressed as the product formed over a particular amount of time from the starting material. In some embodiments, the ethanol yield is calculated as "Gal UD/bushel of corn," reflecting gallon of undenatured ethanol produced per bushel of corn weighting about 56 pounds. In other embodiments, the yield can be represented using other parameters or proxies, for example, in some aspects the yield can be meaningfully reflected in the yield of soluble pentose and/or hexose that can be utilized by the ethanologen.

III. Production of Ethanol from Grain-based Feedstocks

[135] The dry grind ethanol process, also called dry milling process, processes whole corn kernels without steeping and fraction steps. The whole grain is milled, cooked, hydrolyzed, and fermented to produce ethanol.

[136] A simplified diagram of a typical dry grind grain ethanol process is shown in **Figure 1.** At the beginning of this process, the milled grain (e.g., milled corn) enters a slurry tank where it is mixed with process water and enzymes, which typically comprise an alpha amylase, to produce a slurry. The slurry is then gelatinized by the thermostable enzymes in a jet cooker in a liquefaction process. During liquefaction, the mash (e.g., a corn mash) is diluted with the addition of thin stillage, which is also called a backset, prior to fermentation. Fresh water can also be added to supplement the backset or process water streams such as hot condensate from evaporator and thin stillage to the grain slurry tank or to the mash in the liquefaction to give a certain moisture content to the liquefaction mixture, for example, between about 70% to about 80% moisture content. *Dale & Dyner,* (2006) Economic and Technical Analysis of Ethanol Dry Milliing: Model Description. Staff Paper #06-04, Purdue University; *Kwiakowski et al.* (2006) Modeling the process and costs of fuel ethanol production by corn dry grinding process. Ind. Corp. Prod. 23:288-296. The resulting gelatinized mash from liquefaction is rich in dextrin, which can be further hydrolyzed to glucose in saccharification tank by glucoamylases. The glucose rich stream is transferred to a fermentation vessel for ethanol fermentation by yeast. Beer from the fermentation tank is distilled and further dehydrated into a fuel grade ethanol.

[137] The dry grind process also produces highly nutritional co-products which are composed of unhydrolyzed and unfermentated components as well as yeasts at the end of fermentation. The whole slurry containing unfermented solubles and solids that exists from the bottom of the beer column in the distillation system of the dry grind process is called heavy stillage. Heavy stillage is then centrifuged to separate solid and liquid streams.

[138] Wet cake, which is also called distillers' grains (DG) refers to the solids from the centrifugation of heavy stillage. Thin stillage is the liquid setream from the centrifucation of the heavy stillage. It is concentrated by evaoporation to produce syrup which is later mixed with wet cake and dried to produce distillers dry grain with soluble (DDGS). The DG and DDGS are mainly composed of seed hull, germ, proteins and soil and marketed as animal feed due to its high nutritional value.

[139] Thin stillage from the centrifugation is partially recycled as backset. Backset is recycled thin stillage used to prduce slurry in the liquefaction and makes up typically 20-40% of the total water input in the liquefaction. *Dale & Tyner*, (2006). The hot condensate from the evaporator

is also recycled to the evaporator is also recycled to the liquefaction process and mixed with corn meal and alpha amylase enzyme.

[140] Due to its high content of polymeric sugars that have not been utilized during the dry grind process, distillers' grains (DG) have potential not only as an animal feed, but also as an additional source of fermentable sugars to produce more ethanol. The recycle and hydrolysis of distillers' grain as expected benefit dry grind ethanol process without the need to significantly alter or modify the existing dry grind technology and infrastructure.

[141] Recycling of DGs have been accomplished through further additions of enzymes to the DGs as a feedstock. In certain examples, the DGs can be mixed with water or backset to get to an acceptable level of dry solids loading, e.g., about 5% to about 20%, and the mixture can then be pretreated by heating, followed by treatment by a formulation of cellulase such as those commercially available and readily obtained including Spezyme CP (Genencor, Palo Alto, CA), with beta-glucosidase such as Novozyme 188 (Novozymes, Franklin SC), etc. *Kim et al.,* (2008) Bioresour. Technol. 99, 5206. Processes such as these are known to increase the overall yield of ethanol from a given amount of grain-based feedstock.

[142] Other enzymes can also be added in addition or instead of the formulation of cellulase. For example, in order to raise the xylan conversion from such DG materials, xylanase and feruloyesterases have been added to the cellulase mixture, which have led to increased xylose and arabinose yields. The xylose and arabinose can then be fermented by an ethanologen capable of metabolizing C-5 sugars, and as such increasing the overall yield of ethanol even further.

[143] These typical DG recycling processes involve adding cellulases and/or hemicellulases after the initial round of grain to ethanol process which follows the traditional dry grind ethanol process. The cellulases and/or hemicellulases are added in a second saccharification step after the first round of a traditional dry grind grain ethanol process, helping to further break down the lignocellulosic components of the residual materials, which are thought to help release trapped residual starch and/or help producing monomeric sugars that can be fermented into ethanol.

[144] In certain alternative practices, a single saccharification step is employed, whereby cellulases have been added to the saccharification vessel or tank together with a glucoamylase to enhance the overall production of ethanol. See, for example, the disclosures of U.S. Published Patent Application 2012/0276593. Optionally such a saccharification step can be carried out together, completely separately, or overlapping in parts with the fermentation step in such a process. In such instances, small improved overall yield of ethanol can also be achieved.

[145] In a conventional ethanol plant producing ethanol from corn, corn kernels are processed to roughly separate the starch-containing material from other matters, such as fiber and germ. The starch-containing material is then slurried with water and liquefied, in a first enzyme-catalyzed starch liquefaction step, utilizing alpha amylases, that result in the release of glucose from the starch. Alpha-amylases (EC 3.2.1.1) are endohydrolases that randomly cleave internal a-1,4-D-glucosideic bonds. They are capable of degrading the starch slurry to shorter maltodextrines. Often the liquefaction of the starch containing material is done by "cooking" the slurry at a temperature at or near the boiling point of water, but an alternative process is also practiced widely, there the raw starch is converted in the liquefaction step without cooking.

[146] Enzymatic liquefaction can for example involve multiple steps, wherein after the enzyme addition, the slurry is heated to a temperature between about 60 °C and about 95 °C. Then the slurry is further heated, for example, jet-cooked or otherwise, to a temperature of as high as 95 °C to 125 °C, then cooled down to about 60 °C to about 95 °C. More enzyme(s) can then be added, the mash can be held for another 30 minutes to 4 hours at 60 °C to 95 °C. As the alpha amylases degrade the starch, the viscosity of the mixture decreases. In some cases, cellulases are sometimes added to help reduce the viscosity of the mash more effectively. Commercial cellulases including OPTIMASH ™ products such as the BG, TBG, VR or XL product line have been applied for this very purpose. In those liquefaction practices involving "cooking", thermostable alpha amylases, such as alpha amylass from *Bacillus sp.,* are used.

[147] The liquefaction step reduces the viscosity and cleaves the long starch molecules into shorter maltodextrines, but the maltodextrines are typically insufficiently broken down to be readily fermented by yeast to form alcohol. Therefore a further enzymatic step is in most instances necessary to break down the maltodextrins. Glucoamylases and/or maltogenic alpha amylases are used for this second enzymatic step, whereby the enzymes catalyze the hydrolysis of the non-reducing ends of the maltodestrins formed after liquefaction, releasing glucose, malose and isomaltose. Optionallly debranching enzymes such as pullulanases are used to improve saccharfication. The second enzymatic step, also called saccharification step, is typically conducted in an acidic environment (e.g., pH 4) and also at a mildly elevated temperature (e.g., about 60 °C).

[148] The product of the saccharification step is then fermented where the sugar is metabolized by an ethanologen (e.g., yeast) and converted into ethanol. At the end of fermentation, the product is presented as a mixture, or a "beer," comprising a liquid component containing ethanol and water (among other matters) and a solid component containing unfermented particular or solid matters. The beer, including both the solids and liquid components, is then distilled,

during which the fermentation product is processed into ethanol and stillage containing solids in the form of a wet cake. The stillage wet cake can then be dried into distillers dried grains (DDG), whereas the liquid components post distillation can be evaporated and processed into a syrup, and oils contained in the stillage can also be recovered. The water removed from the liquid components, optionally in combination with the water recovered from drying the DDG, can be treated for reuse at the plant, for example, as the water useful for making the grain slurry in the first step of ethanol production.

[149] Although basic enzymatic starch liquefaction processes are well studied and known, it remains the case that most grain-based plant materials, even after milling, liquefaction, saccharification, fermentation and distillation, will continue to retain a significant amount of lignocellulosic matters, which are fibrous and believed to entrap or bind starch, preventing such bound starch from being effectively fermented or otherwise processed.

IV. Lignocellulosic Matters in Grain-Based Feedstocks

[150] It is known that much of the grain-based feedstocks currently used to produce fuel ethanol contain significant amount of lignocellulosic matters. For example, corn kernels are composed of the following matters (*see, Gulati et al.,* (1996). Bioresource Technol. 58:253-64):

Starch	~ 71.7% of dry mass
Soluble sugars	~2.6 % of dry mass
Hemicellulose	~ 5.5% of dry mass
Cellulose	~ 2.4% of dry mass
Lignin	~ 0.2 % of dry mass
Lipids	~ 4.3% of dry mass
Proteins	~ 10.3% of dry mass
Ash	~ 1.4 % of dry mass
Other matters	~ 1.6 % of dry mass

[151] It is also known that distillers' dry grain with soluble can generally contain about 20% or more total glucan, about 16% of the dry mass of which is cellulosic. *See, Youngmi et al.,* (2008), Bioresource Technology, 99:5156-76. It has been estimated that breaking down such cellulosic parts of the grain plant materials can theoretically provide an additional 0.1 gallon of ethanol per bushel of corn, or an increased yield of 0.4-0.5%. *See, Saville et al.,* (2005), Effect of Cellulase Supplementation on Cookline Operation in a Dry Mill Ethanol Plant, 27th Symposium on Biotechnology For Fuels and Chemicals, Denver Colorado. Cellulases have been added in small amounts during liquefaction to decrease the viscosity and aid liquefaction. *See, e.g., Ohgren et al.,*

(2007), Process Biochemistry, 42:834-837. In recent years cellulases have also been included as part of the saccharification mixture in a dry grind grain ethanol plant, for example, in combination with a glucoamylase, to increase the overall ethanol yield. *See,* US Patent Application Publication 2010/0276593. The addition of cellulases, however does not address the much greater component of the grain biomass feedstock, which is hemicellulose.

[152] In a corn kernel, for example, the cellulose and hemicellulose components of the plant feedstock material most likely co-exist in a heterogeneous mixture of complex polysaccharides that interact through covalent and non-covalent means. For example in typical plant cell walls, cellulose (β-1,4 glucan), which generally constitutes 35-50% of carbon found in cell wall components. Cellulose polymers self associate through hydrogen bonding, van der Waals interactions and hydrophobic interactions to form semi-crystalline cellulose microfibrils. These microfibrils also include noncrystalline regions, generally known as amorphous cellulose. The cellulose microfibrils are embedded in a matrix formed of hemicelluloses (including, *e.g.,* xylans, arabinans, and mannans), pectins (*e.g.,* galacturonans and galactans), and various other β-1,3 and β-1,4 glucans. These matrix polymers are often substituted with, for example, arabinose, galactose and/or xylose residues to yield highly complex arabinoxylans, arabinogalactans, galactomannans, and xyloglucans. The hemicellulose matrix is, in turn, surrounded by polyphenolic lignin.

[153] The complexity of the matrix makes it difficult to degrade the fibrous materials solely by the inclusion of cellulases, because hemicellulose components might need to be broken down before the cellulases can act on the core cellulose microfibrils. Lignin constitutes a very small part of a grain-based feedstock, for example, a corn kernel, therefore its permebilization might not be critical for the various enzymes to act on the cellulosic components, but hemicellulose components still needs to be disrupted to allow cellulose-degrading enzymes to act on the cellulose components. Accordingly, a consortium of different enzymatic activities is required to break down both the cellulose and the hemicellose components of a grain-based biomass feedstock, causing effective release of the bound starch as well as the hydrolysis of the hemicellulose and cellulose components into fermentable sugars.

V. Cellulases and Hemicellulases

[154] The general model for cellulose depolymerization to glucose involves a minimum of three distinct enzymatic activities. Endoglucanases cleave cellulose chains internally to shorter chains in a process that increases the number of accessible ends, which are more susceptible to exoglucanase activity than the intact cellulose chains. These exoglucanases (*e.g.,*

cellobiohydrolases) are specific for either reducing ends or non-reducing ends, liberating, in most cases, cellobiose, the dimer of glucose. . The accumulating cellobiose is then subject to cleavage by cellobiases (*e.g.,* β-1,4-glucosidases) to glucose.

[155] Cellulose contains only anhydro-glucose. In contrast, hemicellulose contains a number of different sugar monomers. For instance, aside from glucose, sugar monomers in hemicellulose can also include xylose, mannose, galactose, rhamnose, and arabinose. Hemicelluloses mostly contain D-pentose sugars and occasionally small amounts of L-sugars. Xylose is typically present in the largest amount, but mannuronic acid and galacturonic acid also tend to be present. Hemicelluloses include xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan.

[156] The enzymes and multi-enzyme compositions of the disclosure are useful for saccharification of hemicellulose materials, including, *e.g.,* xylan, arabinoxylan, and xylan- or arabinoxylan-containing substrates. Arabinoxylan is a polysaccharide composed of xylose and arabinose, wherein L-α -arabinofuranose residues are attached as branch-points to a β-(1,4)-linked xylose polymeric backbone.

[157] Most biomass sources, including those grain-based feedstock materials as those described herein, can be rather complex. For example, the grain-based feedstock materials such as corn kernels and other grains can contain cellulose, hemicellulose, pectin, lignin, protein, and ash, among other components. Therefore in order to effectively and efficiently break down the fibrous and cellulosic components of the grain feedstock, an enzyme blend/mixture/consortium comprising the requisite cellulase activities as well as hemicellulase activities are useful. An example of a multi-enzyme blend/composition suitable for use to increase production of ethanol in a conventional grain ethanol plant is one that comprises cellobiohydrolase(s), xylanase(s), endoglucanase(s), β-glucosidase(s), β-xylosidase(s), and, optionally, accessory proteins. The enzyme blend/composition is suitably a non-naturally occurring composition. The multi-enzyme blend/composition can be added to the liquefaction mixture, but it is preferably added to the saccharification mixture enzyme composition is preferably added to the saccharification step.

[158] In certain embodiments, the saccharification step and fermentation step are simultaneous (SSF), and in those instances, the multi-enzyme blend/composition is added to the SSF mixture.

[159] The enzyme blends/compositions of the disclosure can comprise one or more cellulases. Cellulases are enzymes that hydrolyze cellulose (β-1,4-glucan or β D-glucosidic linkages) resulting in the formation of glucose, cellobiose, cellooligosaccharides, and the like. Cellulases have been traditionally divided into three major classes: endoglucanases (EC 3.2.1.4) ("EG"), exoglucanases or cellobiohydrolases (EC 3.2.1.91) ("CBH") and β-glucosidases (β -D-glucoside

glucohydrolase; EC 3.2.1.21) ("BG") (*Knowles et al.,* (1987), Trends in Biotechnology 5(9):255-261; *Shulein,* (1988), Methods in Enzymology, 160:234-242). Endoglucanases act mainly on the amorphous parts of the cellulose fiber, whereas cellobiohydrolases are also able to degrade crystalline cellulose.

[160] Cellulases for use in accordance with the methods and compositions of the disclosure can be obtained from, or produced recombinantly from, *inter alia*, one or more of the following organisms: *Crinipellis scapella*, *Macrophomina phaseolina*, *Myceliophthora thermophila*, *Sordaria fimicola*, *Volutella colletotrichoides*, *Thielavia terrestris*, *Acremonium sp.*, *Exidia glandulosa*, *Fomes fomentarius*, *Spongipellis sp.*, *Rhizophlyctis rosea*, *Rhizomucor pusillus*, *Phycomyces niteus*, *Chaetostylum fresenii*, *Diplodia gossypina*, *Ulospora bilgramii*, *Saccobolus dilutellus*, *Penicillium verruculosum*, *Penicillium chrysogenum*, *Thermomyces verrucosus*, *Diaporthe syngenesia*, *Colletotrichum lagenarium*, *Nigrospora sp.*, *Xylaria hypoxylon*, *Nectria pinea*, *Sordaria macrospora*, *Thielavia thermophila*, *Chaetomium mororum*, *Chaetomium virscens*, *Chaetomium brasiliensis*, *Chaetomium cunicolorum*, *Syspastospora boninensis*, *Cladorrhinum foecundissimum*, *Scytalidium thermophila*, *Gliocladium catenulatum*, *Fusarium oxysporum ssp. lycopersici*, *Fusarium oxysporum ssp. passiflora*, *Fusarium solani*, *Fusarium anguioides*, *Fusarium poae*, *Humicola nigrescens*, *Humicola grisea*, *Panaeolus retirugis*, *Trametes sanguinea*, *Schizophyllum commune*, *Trichothecium roseum*, *Microsphaeropsis sp.*, *Acsobolus stictoideus spej.*, *Poronia punctata*, *Nodulisporum sp.*, *Trichoderma sp.* (*e.g.*, *Trichoderma reesei*) and *Cylindrocarpon sp.*

[161] Cellulases suitable for use in the compositions and methods of the disclosure can be produced by engineering certain microorganisms capable of producing heterologous polypeptides, overproducing endogenous polypeptides, or simply producing recombinantly engineered polypeptides in large amounts. Preferably the microorganism used to produce the cellulases is one that naturally has the capability of such high productivity, but the microorganism may also suitably be one that is engineered to achieve such high productivity. Moreover, the microorganism used to produce the cellulases is one that is capable of secreting the polypeptides it heterologously produces, overproduces, or produces in very large amounts. Accordingly fermentation broths enriched with one or more of the cellulases described herein may be suitable. A number of microorganisms have been extensively studied and improved upon to be useful for producing such cellulases, including, for example, a *Trichoderma, Humicola, Fusarium, Aspergillus, Neurospora, Penicillium, Cephalosporium, Achlya, Podospora, Endothia, Mucor, Cochliobolus, Pyricularia,* or *Chrysosporium.* Suitably the cellulases can be used directly in the form of the fermentation broth, which has not been subject

to, or has only been minimally subject to, post-production processing such as purification, enrichment, filtration, clarification, etc. On the other hand, the cellulases can be used in the form of a cell-free fermentation broth.

[162] For example, a cellulase for use in the method and/or composition of the disclosure is a whole cellulase and/or is capable of achieving at least 0.1 (e.g. 0.1 to 0.4) fraction product as determined by the calcofluor assay described below:

[163] Phosphoric acid swollen cellulose (PASC) can be prepared from Avicel PH-101 using an adapted protocol of *Walseth,* (1971), TAPPI 35:228, and of *Wood,* (1971), Biochem. J. 121:353-362. In an examplified method, Avicel is solubilized in concentrated phosphoric acid then precipitated using cold deionized water. After the cellulose is collected and washed with water to achieve neutral pH, it is diluted to 1% solids in 50 mM sodium acetate buffer, at pH 5.0.

[164] All enzyme dilutions can be made made with a 50 mM sodium acetate buffer, pH 5.0. GC220 Cellulase (Danisco US Inc., Genencor) is diluted to 2.5, 5, 10, and 15 mg protein/g PASC, to produce a linear calibration curve. Samples to be tested are diluted to fall within the range of the calibration curve, *i.e.*, to obtain a response of 0.1 to 0.4 fraction product. One hundred and fifty (150) μL of cold 1% PASC is added to each 20 μL of enzyme solution in suitable vessles, for example, 96-well microtiter plates. The plates are covered and incubated for 2 hrs at 50 °C, spun at 200 rpm, in an incubator/shaker. The reactions are then quenched using 100 μL of 50 μg/mL Calcofluor in 100 mM Glycine, pH 10. Fluorescence is read on a fluorescence microplate reader at excitation wavelength Ex = 365 nm and emission wavelength Em = 435 nm. The result is expressed as the fraction product according to the equation:

FP = 1-(Fl sample - Fl buffer with cellobiose)/(Fl zero enzyme – Fl buffer with cellobiose),

wherein “FP” is fraction product and “Fl” is fluorescence units.

V.1. β-Glucosidase

[165] The enzyme blends/compositions of the disclosure can optionally comprise one or more β-glucosidases. The term "β-glucosidase" as used herein refers to a β-D-glucoside glucohydrolase classified as EC 3.2.1.21, and/or members of certain GH families, including, without limitation, members of GH families 1, 3, 9 or 48, which catalyze the hydrolysis of cellobiose to release β-D-glucose.

[166] Suitable β-glucosidase can be obtained from a number of microorganisms, by recombinant means, or be purchased from commercial sources. Examples of β- glucosidases

from microorganisms include, without limitation, ones from bacteria and fungi. For example, a β-glucosidase of the present disclosure is suitably obtained from a filamentous fungus.

[167] The β-glucosidases can be obtained, or produced recombinantly, from, *inter alia, Aspergillus aculeatus* (*Kawaguchi et al.* (1996) Gene, 173: 287-288), *Aspergillus kawachi* (*Iwashita et al.* (1999) Appl. Environ. Microbiol. 65: 5546-5553), *Aspergillus oryzae* (WO 2002/095014), *Cellulomonas biazotea* (Wong *et al.* Gene, 1998, 207:79-86), *Penicillium funiculosum* (WO 2004/078919), *Saccharomycopsis fibuligera* (*Machida et al.* (1988) Appl. Environ. Microbiol. 54: 3147-3155), *Schizosaccharomyces pombe* (*Wood et al.* (2002) Nature 415: 871-880), or *Trichoderma reesei* (*e.g.,* β-glucosidase 1 (U.S. Patent No. 6,022,725), β-glucosidase 3 (U.S. Patent No.6,982,159), β- glucosidase 4 (U.S. Patent No. 7,045,332), β-glucosidase 5 (US Patent No. 7,005,289), β-glucosidase 6 (U.S. Publication No. 20060258554), β-glucosidase 7 (U.S. Publication No. 20060258554)).

[168] The β-glucosidase can be produced by expressing an endogenous or exogenous gene encoding a β-glucosidase. For example, β-glucosidase can be secreted into the extracellular space *e.g.,* by Gram-positive organisms (*e.g., Bacillus* or *Actinomycetes*), or eukaryotic hosts (*e.g., Trichoderma*, *Aspergillus*, *Saccharomyces*, or *Pichia*). The β-glucosidase can be, in some circumstances, overexpressed or underexpressed.

[169] The β-glucosidase can also be obtained from commercial sources. Examples of commercial β-glucosidase preparation suitable for use in the present disclosure include, for example, *Trichoderma reesei* β-glucosidase in Accellerase® BG (Danisco US Inc., Genencor); NOVOZYM™ 188 (a β-glucosidase from *Aspergillus niger*); *Agrobacterium sp.* β-glucosidase, and *Thermatoga maritima* β-glucosidase from Megazyme (Megazyme International Ireland Ltd., Ireland.).

[170] Moreover, the β-glucosidase can be a component of a whole cellulase, as described in Section V.4 below.

[171] β-glucosidase activity can be determined by a number of suitable means known in the art, such as the assay described by *Chen et al.,* (1992) Biochimica et Biophysica Acta, 121:54-60, wherein 1 pNPG denotes 1 μmoL of Nitrophenol liberated from 4-nitrophenyl-β-D-glucopyranoside in 10 min at 50°C (122°F) and pH 4.8.

V.2. Endoglucanases

[172] The enzyme blends/compositions of the disclosure optionally comprise one or more endoglucanase. Any endoglucanase (EC 3.2.1.4) can be used in the methods and compositions of the present disclosure. An endoglucanse can be produced by expressing an endogenous or

exogenous endoglucanase gene. The endoglucanase can be, in some circumstances, overexpressed or underexpressed.

[173] For example, *Trichoderma reesei* EG1 (*Penttila et al.,* (1986) Gene, 63:103-112) and/or EG2 (*Saloheimo et al.,* (1988) Gene, 63:11-21) are suitably used in the methods and compositions of the present disclosure.

[174] A thermostable *Thielavia terrestris* endoglucanase (*Kvesitadaze et al.,* (1995) Applied Biochem. Biotech. 50:137-143) is, in another example, used in the methods and compositions of the present disclosure. Moreover, a *Trichoderma reesei* EG3 (*Okada et al.* (1988) Appl. Environ. Microbiol. 64:555-563), EG4 (*Saloheimo et al.* (1997) Eur. J. Biochem. 249:584-591), EG5 (*Saloheimo et al.* (1994) Molecular Microbiology, 13:219-228), EG6 (U.S. Patent Publication No. 20070213249), or EG7 (U.S. Patent Publication No. 20090170181), an *Acidothermus cellulolyticus* EI endoglucanase (U.S. Pat. No. 5,536,655), a *Humicola insolens* endoglucanase V (EGV) (Protein Data Bank entry 4ENG), a *Staphylotrichum coccosporum* endoglucanase (U.S. Patent Publication No. 20070111278), an *Aspergillus aculeatus* endoglucanase F1-CMC (*Ooi et al.* (1990) Nucleic Acid Res. 18:5884), an *Aspergillus kawachii* IFO 4308 endoglucanase CMCase-1 (*Sakamoto et al.* (1995) Curr. Genet. 27:435-439), an *Erwinia carotovara* (Saarilahti *et al.* Gene 1990, 90:9-14); or an *Acremonium thermophilum* ALKO4245 endoglucanase (U.S. Patent Publication No. 20070148732) can also be used. Additional suitable endoglucanases are described in, *e.g.,* WO 91/17243, WO 91/17244, WO 91/10732, U.S. Patent No. 6,001,639.

V.3. Cellobiohydrolases

[175] Any cellobiohydrolase (EC 3.2.1.91) ("CBH") can be optionally used in the methods and blends/compositions of the present disclosure. The cellobiohydrolase can be produced by expressing an endogeneous or exogeneous cellobiohydrolase gene. The cellobiohydrolase can be, in some circumstances, overexpressed or under expressed.

[176] For example, *Trichoderma reesei* CBHI (*Shoemaker et al.* (1983) Bio/Technology 1:691-696) and/or CBHII (*Teeri et al.* (1983) Bio/Technology, 1:696-699) can be suitably used in the methods and blends/compositions of the present disclosure.

[177] Suitable CBHs can be selected from an *Agaricus bisporus* CBH1 (Swiss Prot Accession No. Q92400), an *Aspergillus aculeatus* CBH1 (Swiss Prot Accession No. O59843), an *Aspergillus nidulans* CBHA (GenBank Accession No. AF420019) or CBHB (GenBank Accession No. AF420020), an *Aspergillus niger* CBHA (GenBank Accession No. AF156268) or CBHB (GenBank Accession No. AF156269), a *Claviceps purpurea* CBH1 (Swiss Prot

Accession No. O00082), a *Cochliobolus carbonarum* CBH1 (Swiss Prot Accession No. Q00328), a *Cryphonectria parasitica* CBH1 (Swiss Prot Accession No. Q00548), a *Fusarium oxysporum* CBH1 (Cel7A) (Swiss Prot Accession No. P46238), a *Humicola grisea* CBH1.2 (GenBank Accession No. U50594), a *Humicola grisea* var. *thermoidea* CBH1 (GenBank Accession No. D63515) a CBHI.2 (GenBank Accession No. AF123441), or an exo1 (GenBank Accession No. AB003105), a *Melanocarpus albomyces* Cel7B (GenBank Accession No. AJ515705), a *Neurospora crassa* CBHI (GenBank Accession No. X77778), a *Penicillium funiculosum* CBHI (Cel7A) (U.S. Patent Publication No. 20070148730), a *Penicillium janthinellum* CBHI (GenBank Accession No. S56178), a *Phanerochaete chrysosporium* CBH (GenBank Accession No. M22220), or a CBHI-2 (Cel7D) (GenBank Accession No. L22656), a *Talaromyces emersonii* CBH1A (GenBank Accession No. AF439935), a *Trichoderma viride* CBH1 (GenBank Accession No. X53931), or a *Volvariella volvacea* V14 CBH1 (GenBank Accession No. AF156693).

V.4. Whole Cellulases

[178] An enzyme blend/composition of the disclosure can further comprise a whole cellulase. As used herein, a "whole cellulase" refers to either a naturally occurring or a non-naturally occurring cellulase-containing composition comprising at least 3 different enzyme types: (1) an endoglucanase, (2) a cellobiohydrolase, and (3) a β-glucosidase, or comprising at least 3 different enzymatic activities: (1) an endoglucanase activity, which catalyzes the cleavage of internal β-1,4 linkages, resulting in shorter glucooligosaccharides, (2) a cellobiohydrolase activity, which catalyzes an "exo"-type release of cellobiose units (β-1,4 glucose-glucose disaccharide), and (3) a β-glucosidase activity, which catalyzes the release of glucose monomer from short cellooligosaccharides (*e.g.,* cellobiose).

[179] A "naturally occurring cellulase-containing" composition is one produced by a naturally occurring source, which comprises one or more cellobiohydrolase-type, one or more endoglucanase- type, and one or more β-glucosidase-type components or activities, wherein each of these components or activities is found at the ratio and level produced in nature, untouched by the human hand. Accordingly, a naturally occurring cellulase-containing composition is, for example, one that is produced by an organism unmodified with respect to the cellulolytic enzymes such that the ratio or levels of the component enzymes are unaltered from that produced by the native organism in nature. A "non-naturally occurring cellulase-containing composition" refers to a composition produced by: (1) combining component cellulolytic enzymes either in a

naturally occurring ratio or a non-naturally occurring, *i.e.,* altered, ratio; or (2) modifying an organism to overexpress or underexpress one or more cellulolytic enzymes; or (3) modifying an organism such that at least one cellulolytic enzyme is deleted. A "non-naturally occurring cellulase containing" composition can also refer to a composition resulting from adjusting the culture conditions for a naturally-occurring organism, such that the naturally-occurring organism grows under a non-native condition, and produces an altered level or ratio of enzymes. Accordingly, in some embodiments, the whole cellulase preparation of the present disclosure can have one or more EGs and/or CBHs and/or β-glucosidases deleted and/or overexpressed.

[180] In the present disclosure, a whole cellulase preparation can be from any microorganism that is capable of hydrolyzing a cellulosic material. In some embodiments, the whole cellulase preparation is a filamentous fungal whole cellulase. For example, the whole cellulase preparation can be from an *Acremonium, Aspergillus, Emericella, Fusarium, Humicola, Mucor, Myceliophthora, Neurospora, Penicillium, Scytalidium, Thielavia, Tolypocladium,* or *Trichoderma* species. The whole cellulase preparation is, for example,an *Aspergillus aculeatus, Aspergillus awamori, Aspergillus foetidus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger,* or *Aspergillus oryzae* whole cellulase. Moreover, the whole cellulase preparation can be a *Fusarium bactridioides, Fusarium cerealis, Fusarium crookwellense, Fusarium culmorum, Fusarium graminearum, Fusarium graminum, Fusarium heterosporum, Fusarium negundi, Fusarium oxysporum, Fusarium reticulatum, Fusarium roseum, Fusarium sambucinum, Fusarium sarcochroum, Fusarium sporotrichioides, Fusarium sulphureum, Fusarium torulosum, Fusarium trichothecioides*, or *Fusarium venenatum* whole cellulase preparation. The whole cellulase preparation can also be a *Humicola insolens, Humicola lanuginosa, Mucor miehei, Myceliophthora thermophila, Neurospora crassa, Penicillium purpurogenum, Penicillium funiculosum, Scytalidium thermophilum*, or *Thielavia terrestris* whole cellulase preparation. Moreover, the whole cellulase preparation can be a *Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei* (*e.g.,* RL-P37 (*Sheir-Neiss G et al.* (1984) Appl. Microbiol. Biotechnology, 20, pp.46-53), QM9414 (ATCC No. 26921), NRRL 15709, ATCC 13631, 56764, 56466, 56767), or a *Trichoderma viride* (*e.g.,* ATCC 32098 and 32086) whole cellulase preparation.

[181] The whole cellulase preparation can, in particular, suitably be a *Trichoderma reesei* RutC30 whole cellulase preparation, which is available from the American Type Culture Collection as *Trichoderma reesei* ATCC 56765. For example, the whole cellulase preparation can also suitably be a whole cellulase of *Penicillium funiculosum*, which is available from the American Type Culture Collection as *Penicillium funiculosum* ATCC Number: 10446.

[182] The whole cellulase preparation can also be obtained from commercial sources. Examples of commercial cellulase preparations suitable for use in the methods and compositions of the present disclosure include, for example, CELLUCLAST™ and Cellic™ (Novozymes A/S) and LAMINEX™ BG, IndiAge™ 44L, Primafast™ 100, Primafast™ 200, Spezyme™ CP, Accellerase® 1000 and Accellerase® 1500 (Danisco US. Inc., Genencor).

[183] Suitable whole cellulase preparations can be made using any microorganism cultivation methods known in the art, especially fermentation, resulting in the expression of enzymes capable of hydrolyzing a cellulosic material. As used herein, "fermentation" refers to shake flask cultivation, small- or large-scale fermentation, such as continuous, batch, fed-batch, or solid state fermentations in laboratory or industrial fermenters performed in a suitable medium and under conditions that allow the cellulase and/or enzymes of interest to be expressed and/or isolated.

[184] Generally, the microorganism is cultivated in a cell culture medium suitable for production of enzymes capable of hydrolyzing a cellulosic material. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures and variations known in the art. Suitable culture media, temperature ranges and other conditions for growth and cellulase production are known in the art. As a non-limiting example, a typical temperature range for the production of cellulases by *Trichoderma reesei* is 24°C to 28°C

[185] The whole cellulase preparation can be used as it is produced by fermentation with no or minimal recovery and/or purification. For example, once cellulases are secreted into the cell culture medium, the cell culture medium containing the cellulases can be used directly. The whole cellulase preparation can comprise the unfractionated contents of fermentation material, including the spent cell culture medium, extracellular enzymes and cells. On the other hand, the whole cellulase preparation can also be subject to further processing in a number of routine steps, *e.g.,* precipitation, centrifugation, affinity chromatography, filtration, or the like. For example, the whole cellulase preparation can be concentrated, and then used without further purification. The whole cellulase preparation can, for example, be formulated to comprise certain chemical agents that decrease cell viability or kill the cells after fermentation. The cells can, for example, be lysed or permeabilized using methods known in the art.

[186] The endoglucanase activity of the whole cellulase preparation can be determined using carboxymethyl cellulose (CMC) as a substrate. A suitable assay measures the production of reducing ends created by the enzyme mixture acting on CMC wherein 1 unit is the amount of

enzyme that liberates 1 μmoL of product/min (*Ghose, T. K.*, (1987) Pure & Appl. Chem. 59, pp. 257-268).

[187] The whole cellulase can be a β-glucosidase-enriched cellulase. The β-glucosidase-enriched whole cellulase generally comprises a β-glucosidase and a whole cellulase preparation. The β-glucosidase-enriched whole cellulase compositions can be produced by recombinant means. For example, such a whole cellulase preparation can be achieved by expressing a β-glucosidase in a microorganism capable of producing a whole cellulase The β-glucosidase-enriched whole cellulase composition can also, for example, comprise a whole cellulase preparation and a β-glucosidase. For instance, the β-glucosidase-enriched whole cellulase composition can suitably comprise at least 5 wt.%, 7 wt.%, 10 wt.%, 15 wt.% or 20 wt.%, and up to 25 wt.%, 30 wt.%, 35 wt.%, 40 wt.%, or 50 wt.% β-glucosidase based on the total weight of proteins in that blend/composition.

[188] It is generally the case that the more cellulases are added to the saccharification step, up to a certain level when all cellulose components of the grain material that can be digested are digested, the more starch is released and/or the more glucose and other soluble oligo C-6 sugars are produced, hence the more ethanol is produced by the overall process. However, in contrast, while the enzyme blends/compositions of the disclosure can comprise one or more hemicellulases, which can help to break down the hemicellulose components of the grain material, further releasing residual trapped starch, and even provide broken-down soluble sugars that can be consumed by the ethanologen, the correlation of hemicellulase dose with improvements is not as direct. It is noted that at a lower than threshold level of hemicellulase in the saccharification mixture, the viscosity or thickness of the post fermentation mixture as well as that of the thin stillage dramatically increases, so much so that the pressure build up is so severe that the entire ethanol production process must be aborted. The solution to this downstream pressure problem is not to reduce the amount of hemicellulases however, but surprisingly is to increase the dose of hemicellulase, to above a threshold level as described herein.

[189] A wide variety of fungi and bacteria are capable of enzymatically hydrolyzing hemicelluloses. Similar to cellulose degradation, hemicellulose hydrolysis involves coordinated actions of a number of enzymes. Hemicellulases are often grouped into three general categories: the endo-acting enzymes that attack internal bonds within polysaccharide chains, the exo-acting enzymes that act processively from either the reducing or the nonreducing end of the polysaccharide chain, and the accessory enzymes, acetylesterases, and/or esterases that hydrolyze lignin glycoside bonds. Examples of esterases can include coumaric acid esterase and ferulic

acid esterase (*Wong et al.,* (1988), Microbiol. Rev. 52:305-317; *Tenkanen and Poutanen*, (1992) Significance of esterases in the degradation of xylans, in Xylans and Xylanases, *Visser et al.,* eds., Elsevier, New York, N.Y., pp. 203-212; *Coughlan and Hazlewood,* (1993) Hemicellulose and hemicellulases, Portland, London, UK; *Brigham et al.,* (1996) Hemicellulases: Diversity and applications, in Handbook on Bioethanol: Production and Utilization, Wyman, ed., Taylor & Francis, Washington, D.C., pp. 119-141).

[190] Suitable hemicellulases for use with the compositions and/or methods of the present disclosure include, for example, xylanases, arabinofuranosidases, acetyl xylan esterase, glucuronidases, endo-galactanase, mannanases, endo or exo arabinases, exo-galactanases, and mixtures thereof. Examples of endo-acting hemicellulases and ancillary enzymes include, without limitation, endoarabinanase, endoarabinogalactanase, endoglucanase, endomannanase, endoxylanase, and feraxan endoxylanase. Examples of exo-acting hemicellulases and ancillary enzymes include, without limitation, L-α-arabinosidase, L-β-arabinosidase, α-1,2-L-fucosidase, α-D-galactosidase, β-D-galactosidase, β-D-glucosidase, β-D-glucuronidase, β-D-mannosidase, β-D-xylosidase, exoglucosidase, exocellobiohydrolase, exomannobiohydrolase, exomannanase, exoxylanase, xylan α-glucuronidase, and coniferin β-glucosidase. Examples of esterases include, without limitation, acetyl esterases (acetylgalactan esterase, acetylmannan esterase, and acetylxylan esterase) and aryl esterases (coumaric acid esterase and ferulic acid esterase).

[191] In certain aspects, the hemicellulase is an exo-acting hemicellulase. Preferably, the exo-acting hemicellulase has the ability to hydrolyze hemicellulose under acidic conditions, for example, at or below pH 7.

[192] In certain aspects, the hemicellulase is added in an effective amount. For example, the hemicellulase is added to the multienzyme blends of the present disclosure in an amount of about 0.001 wt.% or more, about 0.002 wt.% or more, about 0.0025 wt.% or more, about 0.005 wt.% or more, or about 0.01 wt.% or more relative to the weight of solids in the complete fermentation medium. In another example, the hemicellulase is added to the multienzyme blends of the present disclosure in an amount of about 0.001 wt.% to about 5.0 wt.%, for example, about 0.025 wt.% to about 4.0 wt.%, about 0.005 wt.% to about 2.0 wt.% relative to the weight of solids in the complete fermentation medium.

V.5. Xylanases

[193] The hemicellulases suitable for use with the methods and compositions of the disclosure can comprise one or more xylanases.

[194] The term "xylanase" as used herein refers to any xylanase classified in or under EC 3.2.1.8. Suitable xylanases include, for example, a *Caldocellum saccharolyticum* xylanase (*Luthi et al.,* (1990) Appl. Environ. Microbiol. 56(9):2677-2683), a *Thermatoga maritima* xylanase (*Winterhalter & Liebel*, (1995) Appl. Environ. Microbiol. 61(5):1810–1815), a *Thermatoga Sp.* Strain FJSS-B.1 xylanase (*Simpson et al.,* (1991) Biochem. J. 277, 413-417), a *Bacillus circulans* xylanase (BcX) (U.S. Patent No. 5,405,769), an *Aspergillus niger* xylanase (*Kinoshita et al.,* (1995) J. Ferment. Bioeng. 79(5):422-428); a *Streptomyces lividans* xylanase (*Shareck et al.,* (1991) Gene 107:75-82; *Morosoli et al.,* (1986) Biochem. J. 239:587-592; *Kluepfel et al.,* (1990) Biochem. J. 287:45-50); *Bacillus subtilis* xylanase (*Bernier et al.,* (1983) Gene 26(1):59–65); a *Cellulomonas fimi* xylanase (*Clarke et al.,* (1996) FEMS Microbiol. Lett. 139:27-35), a *Pseudomonas fluorescens* xylanase (*Gilbert et al.,* (1988) J. Gen. Microbiol. 134:3239-3247); a *Clostridium thermocellum* xylanase (*Dominguez et al.,* (1995) Nat. Struct. Biol. 2(7):569-76); a *Bacillus pumilus* xylanase (*Nuyens et al.,* (2001) Appl. Microbiol. Biotech. 56:431-434; *Yang et al.,* (1988) Nucleic Acids Res. 16(14B):7187); a *Clostridium acetobutylicum* P262 xylanase (*Zappe et al.,* (1990) Nucleic Acids Res. 18(8):2179) or a *Trichoderma harzianum* xylanase (*Rose et al.,* (1987) J. Mol. Biol. 194(4):755–756).

[195] Xylanases can suitably be obtained from a number of sources, including, for example, fungal and bacterial organisms, such as *Aspergillus*, *Disporotrichum*, *Penicillium*, *Neurospora*, *Fusarium*, *Trichoderma*, Humicola, *Thermomyces*, and *Bacillus*. Certain commercially available preparations comprising xylanase(s) can also be used in the compositions and methods of the present disclosure; those include Multifect® xylanase, Laminex® BG and Spezyme® CP (Danisco US, Genencor), and Celluclast® and Viscozyme® (Novozymes A/S).

[196] The xylanase can be produced by expressing an endogenous or exogenous gene encoding a xylanase. The xylanase can be, in some circumstances, overexpressed or underexpressed. In certain embodiments, the xylanases can be co-expressed or part of a hemicellulase mixture comprising one or more other hemicellulases, optionally also one or more other celllases and/or other enzymes such as accessory enzymes.

V.6. β-Xylosidases

[197] The hemicellulases suitable for use with the methods and compositions of the disclosure can comprise one or more β-xylosidases.

[198] As used herein, the term "β-xylosidase" refers to any β-xylosidase classified in or under EC 3.2.1.37. Suitable β-xylosidases include, for example *Talaromyces emersonii* Bxl1 (*Reen et al.*, (2003) Biochem. Biophys. Res. Commun. 305(3):579-85); as well as β-xylosidases obtained

from *Geobacillus stearothermophilus* (*Shallom et al.,* (2005) Biochem. 44:387-397); *Scytalidium thermophilum* (*Zanoelo et al.,* (2004) J. Ind. Microbiol. Biotechnol. 31:170-176); *Trichoderma lignorum* (*Schmidt,* (1988) Methods Enzymol. 160:662-671); *Aspergillus awamori* (*Kurakake et al.,* (2005) Biochim. Biophys. Acta 1726:272-279); *Aspergillus versicolor* (*Andrade et al.,* Process Biochem. 39:1931-1938); *Streptomyces sp.* (*Pinphanichakarn et al.,* (2004) World J. Microbiol. Biotechnol. 20:727-733); *Thermotoga maritima* (*Xue and Shao,* (2004) Biotechnol. Lett. 26:1511-1515); *Trichoderma sp.* SY (*Kim et al.,* (2004) J. Microbiol. Biotechnol. 14:643-645); *Aspergillus niger* (*Oguntimein and Reilly,* (1980) Biotechnol. Bioeng. 22:1143-1154); or *Penicillium wortmanni* (*Matsuo et al.,* (1987) Agric. Biol. Chem. 51:2367-2379).

[199] The β-xylosidase can be produced by expressing an endogenous or exogenous gene encoding a β-xylosidase. The β-xylosidase can be, in some circumstances, overexpressed or underexpressed by a suitable host cell. In some embodiments, the one or more β-xylosidases can be co-expressed or part of a hemicellulase mixture comprising one or more other hemicellulases, optionally also one or more other celllases and/or other enzymes such as accessory enzymes.

V.7. L-α-Arabinofuranosidases

[200] The hemicellulases suitable for use with the methods and compositions of the disclosure can comprise one or more L-α-arabinofuranosidases.

[201] As used herein, the term "L-α-arabinofuranosidase" refers to any enzyme classified in or under EC 3.2.1.55. For example, they can be GH43 or GH51 enzymes. Suitable L-α-arabinofuranosidase can be obtained from, for example, *Aspergillus oryzae* (*Numan & Bhosle,* (2006) J. Ind. Microbiol. Biotechnol. 33:247-260); *Aspergillus sojae* (*Oshima et al.,* (2005) J. Appl. Glycosci. 52:261-265); *Bacillus brevis* (*Numan & Bhosle*, (2006) J. Ind. Microbiol. Biotechnol. 33:247-260); *Bacillus stearothermophilus* (*Kim et al.,* (2004) J. Microbiol. Biotechnol. 14:474-482); *Bifidobacterium breve* (*Shin et al.,* (2003) Appl. Environ. Microbiol. 69:7116-7123; *Bifidobacterium longum* (*Margolles et al.,* (2003) Appl. Environ. Microbiol. 69:5096-5103); *Clostridium thermocellum* (*Taylor et al.,* (2006) Biochem. J. 395:31-37); *Fusarium oxysporum* (*Panagiotou et al.,* (2003) Can. J. Microbiol. 49:639-644); *Fusarium oxysporum f. sp. dianthi* (*Numan & Bhosle,* (2006) J. Ind. Microbiol. Biotechnol. 33:247-260); *Geobacillus stearothermophilus* T-6 (*Shallom et al.,* (2002) J. Biol. Chem. 277:43667-43673); *Hordeum vulgare* (*Lee et al.,* (2003) J. Biol. Chem. 278:5377-5387); *Penicillium chrysogenum* (*Sakamoto et al.,* (2003) Biophys. Acta 1621:204-210); *Penicillium sp.* (*Rahman et al.,* (2003) Can. J. Microbiol. 49:58-64); *Pseudomonas cellulosa* (*Numan & Bhosle,* (2006) J. Ind.

Microbiol. Biotechnol. 33:247-260); *Rhizomucor pusillus* (*Rahman et al.,* (2003) Carbohydr. Res. 338:1469-1476) ; *Streptomyces chartreusis* (*Numan & Bhosle,* (2006) J. Ind. Microbiol. Biotechnol. 33:247-260); *Streptomyces thermoviolacus* (*Numan & Bhosle,* (2006) J. Ind. Microbiol. Biotechnol. 33:247-260); *Thermoanaerobacter ethanolicus* (*Numan & Bhosle*, (2006) J. Ind. Microbiol. Biotechnol. 33:247-260); *Thermobacillus xylanilyticus* (*Numan & Bhosle,* (2006) J. Ind. Microbiol. Biotechnol. 33:247-260); *Thermomonospora fusca* (*Tuncer & Ball,* (2003) Folia Microbiol. (Praha) 48:168-172); *Thermotoga maritima* (*Miyazaki,* (2005) Extremophiles 9:399-406); *Trichoderma sp.* SY (*Jung et al.,* (2005) Agric. Chem. Biotechnol. 48:7-10); *Aspergillus kawachii* (*Koseki et al.,* (2006) Biochim. Biophys. Acta 1760:1458-1464); *Fusarium oxysporum f. sp. dianthi* (*Chacon-Martinez et al.,* (2004) Physiol. Mol. Plant Pathol. 64:201-208); *Thermobacillus xylanilyticus* (*Debeche et al.,* (2002) Protein Eng. 15:21-28); *Humicola insolens* (*Sorensen et al.,* (2007) Biotechnol. Prog. 23:100-107); *Meripilus giganteus* (*Sorensen et al.,* (2007) Biotechnol. Prog. 23:100-107); or *Raphanus sativus* (*Kotake et al.,* (2006) J. Exp. Bot. 57:2353-2362).

[202] The L-α-arabinofuranosidase can be produced by expressing an endogenous or exogenous gene encoding an L-α-arabinofuranosidase. The L-α-arabinofuranosidase can be, in some circumstances, overexpressed or underexpressed by a suitable host cell. In some embodiments, the one or more L-α-arabinofuranosidase can be co-expressed or part of a hemicellulase mixture comprising one or more other hemicellulases, optionally also one or more other celllases and/or other enzymes such as accessory proteins or enzymes.

V.8. Accessory proteins

[203] Optionally the saccharification mixture can comprise enzymes other than glucoamylase(s) and the hemicellulases, optionally the cellulases. A number of polypeptides that are known to be cellulosic accessory activities can also be used in conjunction with the methods and compositions herein to further increase or improve the yield of grain ethanol. *See, e.g., Brigham et al.,* (1995) in Handbook on Bioethanol (Charles E. Wyman, ed.), pp. 119-141, *Taylor & Francis,* Washington D.C.; Lee, (1997) J. Biotechnol. 56: 1-24).

[204] The optimum amounts of such accessory enzymes may depend on a number of factors including, without limitation, the specific mixture of component cellulases and hemicellulases, the particular cellulosic and hemicellulosic compositions of the grain-based substrate, the temperature, time, and pH of the saccharification mixture, and the nature of the fermenting organism.

[205] Examples of accessory proteins include, without limitation, mannanases (*e.g.,* endomannanases, exomannanases, and β-mannosidases); galactanases (*e.g.,* endo- and exo-galactanases), arabinases (*e.g.,* endo-arabinases and exo-arabinases), ligninases, amylases, glucuronidases, proteases, esterases (*e.g.,* ferulic acid esterases, acetyl xylan esterases, coumaric acid esterases or pectin methyl esterases), lipases, glycoside hydrolase Family 61 polypeptides, xyloglucanases, CIP1, CIP2, swollenin, expansins, and cellulose disrupting proteins. Examples of accessory proteins can also include CIP1-like proteins, CIP2-like proteins, cellobiose dehydrogenases and manganese peroxidases. Certain of these accessory enzymes such as mannanases, galactanases, arabinases, and esterases are sometimes categorized as hemicellulases.

V.9. Other grain-to-ethanol enzymes

V.9.1. Alpha-Amylases

[206] Any alpha-amylases useful in liquefaction and/or saccharification of starch substrates are contemplated for use herein. Particularly useful are those displaying relatively high thermostability and thus capable of liquefying starch at a temperature above 80°C. Alpha-amylases suitable for the liquefaction process may be from fungal or bacterial origin, particularly alpha-amylases isolated from thermophilic bacteria, such as *Bacillus* species. These *Bacillus* alpha-amylases are commonly referred to as "Termamyl-like alpha-amylases." Well-known Termamyl-like alpha-amylases include those from *B. licheniformis*, *B. amyloliquefaciens*, and *Geobacillus stearothermophilus* (previously known as *Bacillus stearothermophilus*). Other Termamyl-like alpha-amylases include those derived from *Bacillus sp.* NCIB 12289, NCIB 12512, NCIB 12513, and DSM 9375, which are disclosed in WO 95/26397. Contemplated alpha-amylases may also derive from *Aspergillus* species, e.g., *A. oryzae* and *A. niger* alpha-amylases. In addition, commercially available alpha-amylases and products containing alpha-amylases include TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME® SC and SAN™ SUPER (Novozymes A/S, Denmark), and SPEZYME® XTRA, GC 358, SPEZYME® FRED, SPEZYME® FRED-L, and SPEZYME® HPA (Danisco US Inc., Genencor Division).

[207] Alpha-amylases useful herein include wild-type (or parent) enzymes, as well as variants of the parent enzyme. Such variants may have about 80% to about 99% sequence identity to a Termamyl-like alpha-amylase or other wild-type amylase such as the *Bacillus licheniformis* alpha-amylase (disclosed in US 2009/0238923, filed Nov. 3, 2008) or *Geobacillus*

stearothermophilus alpha-amylase (disclosed in US 2009/0252828, filed Nov. 3, 2008). Amylase variants disclosed in WO 96/23874, WO 97/41213, and WO 99/19467 are also contemplated for use herein, including the *Geobacillus stearothermophilus* alpha-amylase variant having the mutations Δ(181-182)+N193F compared to the wild-type alpha-amylase disclosed in WO 99/19467.

[208] In some embodiments, a variant alpha-amylase may display one or more altered properties compared to those of the parent enzyme. The altered properties may advantageously enable the variant alpha-amylase to perform effectively in liquefaction. Similarly, the altered properties may result in improved performance of the variant compared to its parent. These properties may include substrate specificity, substrate binding, substrate cleavage pattern, thermal stability, pH/activity profile, pH/stability profile, stability towards oxidation, stability at lower levels of calcium ion (Ca^{2+}), and/or specific activity. Representative alpha-amylase variants include those disclosed in US 2008/0220476, published Sept. 11, 2008; US 2008/0160573, published July 3, 2008; US 2008/0153733, published June 26, 2008; and US 2008/0083406, published Apr. 10, 2008. Blends of two or more alpha-amylases, each of which may have different properties are also contemplated for use herein.

[209] Alpha-amylase activity may be determined according to the method disclosed in U.S. Patent No.5,958,739, with minor modifications. In brief, the assay uses p-nitrophenyl maltoheptoside (PNP-G_7) as the substrate with the non-reducing terminal sugar chemically blocked. PNP- G_7 can be cleaved by an endo-amylase, for example alpha-amylase. Following the cleavage, an alpha-glucosidase and a glucoamylase digest the substrate to liberate free PNP molecules, which display a yellow color and can be measured by visible spectrophotometry at 410 nm. The rate of PNP release is proportional to alpha-amylase activity. The alpha-amylase activity of a sample is calculated against a standard control.

[210] Variant or mutant alpha-amylases can also be made by the skilled artisan for use herein, beginning for example with any known wild-type sequence. Many methods for making such variants, e.g. by introducing mutations into known genes, are well known in the art. The DNA sequence encoding a parent α-amylase may be isolated from any cell or microorganism producing the α-amylase in question, using various methods well known in the art.

V.9.2. Glucoamylases

[211] Another enzyme contemplated for use in the starch processing, especially during saccharification, is a glucoamylase (EC 3.2.1.3). Glucoamylases are commonly derived from a microorganism or a plant. For example, glucoamylases can be of fungal or bacterial origin.

[212] Exemplary fungal glucoamylases are *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984), *EMBO J.* 3(5): 1097-1102), or variants thereof, such as disclosed in WO 92/00381 and WO 00/04136; *A. awamori* glucoamylase (WO 84/02921); *A. oryzae* glucoamylase (*Agric. Biol. Chem.* (1991), 55(4): 941-949), or variants or fragments thereof. Other contemplated *Aspergillus* glucoamylase variants include variants with enhanced thermal stability: G137A and G139A (Chen et al. (1996), *Prot. Eng.* 9: 499-505); D257E and D293E/Q (Chen et al. (1995), *Prot. Eng.* 8: 575-582); N182 (Chen et al. (1994), *Biochem. J.* 301: 275-281); disulphide bonds, A246C (Fierobe et al. (1996), *Biochemistry*, 35: 8698-8704); and introduction of Pro residues in positions A435 and S436 (Li *et al.* (1997) *Protein Eng.* 10: 1199-1204).

[213] Exemplary fungal glucoamylases may also include *Trichoderma reesei* glucoamylase and its homologues as disclosed in U.S. Patent No. 7,413,879 (Danisco US Inc., Genencor Division). Glucoamylases may include, for example, *T. reesei* glucoamylase, *Hypocrea citrina* var. *americana* glucoamylase, *H. vinosa* glucoamylase, *H. gelatinosa* glucoamylase, *H. orientalis* glucoamylase, *T. konilangbra* glucoamylase, *T. harzianum* glucoamylase, *T. longibrachiatum* glucoamylase, *T. asperellum* glucoamylase, and *T. strictipilis* glucoamylase.

[214] Other glucoamylases contemplated for use herein include *Talaromyces* glucoamylases, in particular derived from *T. emersonii* (WO 99/28448), *T. leycettanus* (U.S. Patent No. RE 32,153), *T. duponti*, or *T. thermophilus* (U.S. Patent No. 4,587,215). Contemplated bacterial glucoamylases include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP 135138) and *C. thermohydrosulfuricum* (WO 86/01831).

[215] Suitable glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase having about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, or even about 90% identity to the amino acid sequence disclosed in WO 00/04136. Suitable glucoamylases may also include the glucoamylases derived from *T. reesei*, such as a glucoamylase having about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, or even about 90% identity to the amino acid sequence disclosed in WO 08/045489 (Danisco US Inc., Genencor Division). *T. reesei* glucoamylase variants with altered properties, such as those disclosed in WO 08/045489 and US 2009/0275080, filed Nov. 20, 2008 (Danisco US Inc., Genencor Division), may be particularly useful.

[216] Also suitable are commercial glucoamylases, such as Spirizyme® Fuel, Spirizyme® Plus, and Spirizyme® Ultra (Novozymes A/S, Denmark), G-ZYME® 480, G-ZYME® 480 Ethanol, GC 147, DISTILLASE®, and FERMENZYME® (Danisco US Inc., Genencor

Division). Glucoamylases may be added in an amount of about 0.02-2.0 GAU/g ds or about 0.1-1.0 GAU/g ds, e.g., about 0.2 GAU/g ds.

V.9.3. Pectinases

[217] Pectinases, or pectic enzymes include several different enzymes, for example pectolyase, pectozyme, pectinesterase, and polygalacturonase. Protopectinases can also be considered as pectinases for purposes herein. EC classes that include pectinases are at least EC 3.1.1.11 (pectin methyl esterase), 3.2.1.15 (polygalacturonase), 3.2.1.67 (exopolygalacturonase), 3.2.1.82 (exo-poly-α-galacturonosidase), 4.2.2.2 (pectic lyase), 4.2.2.9 (pectate disaccharide-lyase), 4.2.2.6 (oligogalacturonide lyase), and 4.2.2.10 (pectin lyase). Any of the foregoing alone or in any combination thereof may be used in accordance with the improved saccharification processes provided herein.

[218] Commercial sources of pectinase enzymes include PANZYM (C.H. Boehringer Sohn (Ingelheim, West Germany)), ULTRAZYME (Ciba-Geigy, A.G. (Basel, Switzerland)), PECTOLASE (Grinsteelvaeket (Aarthus, Denmark)), SCLASE (Kikkoman Shoyu, Co. (Tokyo, Japan)), PECTINEX (Schweizerische Ferment, A.G. (Basel, Switzerland)), RAPIDASE (Societe Rapidase, S.A. (Seclin, France)), KLERZYME (Clarizyme Wallerstein, Co. (Des Plaines, USA)), PECTINOL/ROHAMENT (Rohm, GmbH (Darmstadt, West Germany)), and PECTINASE (Biocon Pvt Ltd (Bangalore, India))

V.9.4. Phytases

[219] Phytases are useful for the present disclosure as they are capable of hydrolyzing phytic acid under the defined conditions of the incubation and liquefaction steps. In some embodiments, the phytase is capable of liberating at least one inorganic phosphate from an inositol hexaphosphate (phytic acid). Phytases can be grouped according to their preference for a specific position of the phosphate ester group on the phytate molecule at which hydrolysis is initiated (e.g., as 3-phytases (EC 3.1.3.8) or as 6-phytases (EC 3.1.3.26)). A typical example of phytase is myo-inositol-hexakiphosphate-3-phosphohydrolase.

[220] Phytases can be obtained from microorganisms such as fungal and/or bacterial organisms. Some of these microorganisms include e.g. *Aspergillus* (e.g., *A. niger*, *A. terreus*, *A. ficum,* and *A. fumigatus*), *Myceliophthora (M. thermophila*), *Talaromyces (T. thermophilus*), *Trichoderma spp* (*T. reesei*), and *Thermomyces* (WO 99/49740). Phytases are also available from *Penicillium* species, e.g., *P. hordei* (ATCC No. 22053), *P. piceum* (ATCC No. 10519), or *P. brevi-compactum* (ATCC No. 48944). *See, e.g.*, U.S. Patent No. 6,475,762. In addition,

phytases are available from *Bacillus* (e.g., *B. subtilis*, *Pseudomonas*, *Peniophora*, *E. coli*, *Citrobacter*, *Enterobacter*, and *Buttiauxella* (*see* WO2006/043178)).

[221] Commercial phytases are available such as NATUPHOS (BASF), RONOZYME P (Novozymes A/S), PHZYME (Danisco A/S, Diversa), and FINASE (AB Enzymes). The Maxaliq™ ONE (Danisco US Inc., Genencor Division) blend contains a thermostable phytase that is capable of efficiently reducing viscosity of the liquefact and breaking down phytic acid. The method for determining microbial phytase activity and the definition of a phytase unit has been published by Engelen et al. (1994) *J. of AOAC Int.*, 77: 760-764. The phytase may be a wild-type phytase, a variant, or a fragment thereof.

[222] In one embodiment, the phytase is one derived from the bacterium *Buttiauxiella* spp. The *Buttiauxiella* spp. includes *B. agrestis*, *B. brennerae*, *B. ferragutiase*, *B. gaviniae*, *B. izardii*, *B. noackiae*, and *B. warmboldiae*. Strains of *Buttiauxella* species are available from DSMZ, the German National Resource Center for Biological Material (Inhoffenstrabe 7B, 38124 Braunschweig, Germany). *Buttiauxella* sp. strain P1-29 deposited under accession number NCIMB 41248 is an example of a particularly useful strain from which a phytase may be obtained and used according to the present disclosure. In some embodiments, the phytase is BP-wild-type, a variant thereof (such as BP-11) disclosed in WO 06/043178, or a variant as disclosed in US 2008/0220498. For example, a BP-wild-type and variants thereof are disclosed in Table 1 of WO 06/043178.

V.9.5. Beta-Amylases, Debraching Enzymes, and Others

[223] In another aspect, the use of a beta-amylase is also contemplated. Beta-amylases (EC 3.2.1.2) are exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-α-glucosidic linkages in amylose, amylopectin, and related glucose polymers, thereby releasing maltose. Beta-amylases have been isolated from various plants and microorganisms (Fogarty et al., *Progress in Industrial Microbiology*, Vol. 15, pp. 112-115, 1979). These beta-amylases are characterized by having optimum temperatures in the range from about 40°C to about 65°C, and optimum pH in the range from about 4.5 to about 7.0. Contemplated β-amylases include, but are not limited to, beta-amylases from barley Spezyme® BBA 1500, Spezyme® DBA, Optimalt™ ME, Optimalt™ BBA (Danisco US Inc., Genencor Division); and Novozym™ WBA (Novozymes A/S).

[224] Another enzyme that can optionally be added is a debranching enzyme, such as an isoamylase (EC 3.2.1.68) or a pullulanase (EC 3.2.1.41). Isoamylase hydrolyses α-1,6-D-glucosidic branch linkages in amylopectin and β-limit dextrins and can be distinguished from

pullulanases by the inability of isoamylase to attack pullulan and by the limited action of isoamylase on α-limit dextrins. Debranching enzymes may be added in effective amounts well known to the person skilled in the art.

VI. Use of Hemicellulase in the Production of Ethanol from Grain-Based Feedstock

[225] The complexity of multi-enzyme blends/compositions comprising all the requisite cellulase and hemicellulase activities make these enzyme compositions difficult to produce at low cost. As a result, existing commercial products that are such multi-enzyme blends/compositions remain very expensive, at least relative to the costs of enzymes used in traditional grain ethanol industry such as alpha amylases and/or glucoamylases .

[226] On the other hand, while there is more hemicellulose than cellulose in grain-based feedstock, those components, even combined, constitutes a relatively small percent dry weight of the grain-based feedstock, especially when compared to that of the starch. Thus, and as has been observed, adding small amounts of certain commercial cellulases to the saccharification mixture increases the ethanol yield without significantly adding to the cost of enzymes used in the process, making this practice a worthwhile tradeoff commercially.

[227] The study of cellulosic conversion has informed that cellose breaks down more efficiently in the presence of both hemicellulases and cellulases, as compared to when there is only cellulase present. Accordingly it was reasonably extrapolated that, at the same total protein dose, adding a hemicellulase/cellulase blend may potentially achieve better and more effective breakdown of the fibrous materials, therefore releasing the entrapped starch, making it available for saccharification and fermentation, and also potentially releasing soluble monomeric and small oligomeric cellulosic and hemicellulosic sugars that can be fermented. At the very least, if the same amount of cellulases is included in the saccharification mixture, any addition of hemicellulases would provide a boost in cellulose breakdown, releasing more entrapped or bound starch.

[228] In practice however, it was noted that hemicellulases when added to the saccharification mixture at a level below certain threshold, the downstream pressure buildup and viscosity increases in the post-distillation mixture and thin stillage threatens the integrity of the process to such an extent the small increases in ultimate ethanol yields, if there is any, cannot justify the cost of even introducing the hemicellulases to the process at all. Thus, the added hemicellulases at the levels below threshold might have in fact provides a synergistic boost in breaking the fibrous cellulosic materials that entrap or bind starch, thereby releasing the starch for saccharification and fermentation, but that synergistic improvement will likely be lost and will

unlikely lead to a successful increase in ethanol yield, contrary to what may have been reasonably projected.

[229] The solution to the problematic increase in downstream pressure in the system is not resolved by reducing the dose of hemicellulases in the saccharification mixture even though at below the threshold level, the more hemicellulases are present in the saccharification mixture, the more viscose the thin stillage and the faster the viscosity and pressure buildup. Surprisingly the solution is to increase hemicellulase dosing to a level that is above a certain threshold, for example, to at least about 0.1 mg/g DS, for example, at least about 0.2 mg/g, at least about 0.5 mg/g, at least about 0.7 mg/g, at least about 1.0 mg/g, at least about 1.2 mg/g, at least about 1.5 mg/g, or even at least about 2.0 mg/g DS. Therefore provided herewith is a method of using hemicellulases, optionally together with cellulases, during the saccharification step, optionally in an SSF step, to increase the overall yield of ethanol from a grain-based feedstock, e.g., corn kernel, without increasing the viscosity of the post-distillation mixture and/or of the thin stillage.

[230] Preferably the amount of hemicellulases in the saccharification mixture is about 0.1 mg/g DS to about 2.5 mg/g DS, for example, about 0.3 mg/g DS to about 2.2 mg/g DS, or about 0.5 mg/g DS to about 1.5 mg/g DS.

VII. Improved Saccharification Methods

[231] Following liquefaction, the mash or liquefact is further hydrolyzed through saccharification to produce low molecular weight soluble "fermentable" sugars (DP1, DP2), which can be readily fermented by a suitable ethanologen or fermenting microorganism. In some embodiments, a pre-saccharification step of 1-4 hours may be included between the liquefaction step and the saccharification step. During saccharification, the hydrolysis is generally accomplished enzymatically by the presence of a glucoamylase. In many typical embodiments, an alpha glucosidase and/or an acid alpha-amylase may also be included in the saccharification step. In certain embodiments, cellulases are added to the saccharification along with the glucoamylase to help boost the ethanol yield.

[232] In the improved saccharification method provided herein, hemicellulases are further added to the saccharification mixture. Accordingly the saccharification step is conducted in the presence of glucoamylase, optionally an alpha glucosidase and/or an acid alpha-amylase, a cellulase and/or a cellulase mixture as well as a hemicellulase or hemicellulase mixture.

[233] In one aspect, improved methods of saccharifying a starch-containing substrate, such as a cereal grain or other starchy crop are provided. The methods are useful for preparing a fermentation feedstock. The methods comprise identifying a liquefied starch slurry (liquefact)

that contains at least some lignocellulosic materials; contacting the liquefact with a glucoamylase, a cellulase and a hemicellulase under conditions sufficient for enzyme activity; an allowing time for the enzymatically catalyzed saccharification to occur. A fermentation feedstock is thus produced by the method and is useful for fermentation to produce a fermentation product (e.g., an industrial chemical, a pharmaceutical, an ethanol or other biofuel) Such a fermentation feedstock produces a higher level of an intended fermentation product as compared to one produced from a comparable liquefact saccharified with an enzyme mixture comprising less or no hemicellulase.

[234] In one embodiment, the enzyme activity, particularly the activity of the hemicellulase is sufficient to at least: (a) increase concentration of at least one fermentable sugar in the fermentation feedstock; (b) release at least one starch chain bound to or trapped by the lignocelluloses fibrous materials in the starch-containing substrate; or (c) to achieve sufficient hydrolysis of the hemicellulosic portion of the lignocellulosic material such that there is no detectable level of viscosity or thickness increase in the thin stillage or increased pressure post distillation. Experiments can be conducted wherein any of (a), (b) or (c) above may be measured relative to a control liquefact not contacted with or treated with hemicellulase or at a lower level of hemicellulase in the saccharification step.

VIII. The Ethanologens

[235] The microorganism used in fermentations will depend on the desired end product. Typically, if ethanol is the desired end product, yeast will be used as the fermenting organism.

[236] The term "fermenting microorganism," as used herein, refers to any microorganism suitable for use in a desired fermentation process. Suitable fermenting microorganisms according to the instant disclosure are able to ferment, *i.e.*, convert, sugars, such as, for example, glucose, xylose, arabinose, mannose, galactose, or oligosaccharides, directly or indirectly into the desired fermentation product.

[237] Examples of suitable fermenting microorganisms include, without limitation, fungal organisms, such as yeast. Specifically, a suitable yeast can be selected from strains of the *Saccharomyces spp.*, and in particular, *Saccharomyces cerevisiae*. Various types of yeast are commercially available, among which, for example, ETHANOL RED™ yeast (available from Fermentis/Lesaffre, USA), FALI (available from Fleischmann's Yeast, USA), SUPERSTART™ and THERMOSACC® fresh yeast (available from Ethanol Technology, WI, USA), BIOFERM AFT and XR (available from NABC--North American Bioproducts Corporation, GA, USA),

GERT STRAND (available from Gert Strand AB, Sweden), or FERMIOL (available from DSM Specialties) can be employed in performing the methods of the invention described herein.

[238] In some embodiments, the ethanol-producing microorganism is a yeast, and specifically *Saccharomyces*, such as strains of *S. cerevisiae* (U.S. Patent No. 4,316,956). In other aspects, the yeast is a *Saccharomyces distaticus* or a *Saccharomyces uvarum.* In yet other aspects, the yeast is a *Kluyveromyces*. Non-limiting examples of *Kluvermoyces* include *Kluyveromyces marxianus* or *Kluyveromyces fragilis*. In yet other aspects, the yeast is a *Candida*. Non-limiting examples of *Candida* include *Candida pseudotropicalis* and *Candida brassicae*. In yet other aspects, the yeast is a *Clavispora*. Non-limiting examples of *Clavispora* include *Clavispora lusitaniae* and *Clavispora opuntiae*. In another aspect, the yeast is a *Pachysolen*, *e.g.*, a *Pachysolen tannophilus*. In another aspect, the yeast is a *Bretannomyces*, *e.g.*, a *Bretannomyces clausenii*. Yeast fermentation has been described in the literature. *See, e.g., Philippidis,* (1996) Cellulose bioconversion technology, in Handbook on Bioethanol: Production and Utilization (Wyman, ed., *Taylor & Francis,* Washington, D.C., 179-212).

[239] The amount of starter yeast employed in the methods is an amount effective to produce a commercially significant amount of ethanol in a suitable amount of time, (e.g. to produce at least 10% ethanol from a substrate having between 25-40% DS in less than 72 hours). Yeast cells can be supplied in amounts of about 10^4 to 10^{12}, and typically from about 10^7 to 10^{10} viable yeast count per ml of fermentation broth. The fermentation will include in addition to a fermenting microorganisms (e.g., yeast), nutrients, optionally additional enzymes, including but not limited to phytases. The use of yeast in fermentation is well known. *See, e.g.*, THE ALCOHOL TEXTBOOK, K.A. JACQUES ET AL., EDS. 2003, NOTTINGHAM UNIVERSITY PRESS, UK.

[240] Bacteria that can efficiently ferment glucose to ethanol include, for example, *Zymomonas mobilis* and *Clostridium thermocellum* (*see, e,g., Philippidis,* (1996) *supra*). Alternatively the ethanologen can be one that has genetically manipulated by pathway engineering to introduce an added capability to metabolize, ferment and convert to ethanol also xylose and/or even arabinose in addition to the traditional C6 glucose sugars. *See, e.g., Byoungjin Kim et al.,* (2012) Combinatorial Design of a Highly Efficient Xylose Utilizing Pathway for Cellulosic Biofuels Production in Saccharomyces cerevisiae, Applied and Environmental Microbiology, doi: 10.1128/ AEM. 02736-12, November 2012; *Bärbel Hahn-Hägerdal, et al.,* (2010) Journal of Biotechnology, 150: Suppl.1 (S10); *Lawford HG & Rousseau JD,* (2002) App. Biochem. Biotechnol. 98-100:429-48 (2002).

[241] The cloning of heterologous genes in a *Saccharomyces cerevisiae* (*see, e.g., Chen and Ho,* (1993), Appl. Biochem. Biotechnol. 39-40:135-147; *Ho et al.,* (1998) Appl. Environ.

Microbiol. 64:1852-1859), or in a bacterium such as an *Escherichia coli* (*see, e.g., Beall et al.,* (1991) Biotech. Bioeng. 38: 296-303), a *Klebsiella oxytoca* (*see, e.g., Ingram, et al.,* (1998) Biotechnol. Bioeng. 58:204-214), or a *Zymomonas mobilis* (*see, e.g., Zhang et al.,* (1995) Science 267:240-243; *Deanda et al.,* (1996) Appl. Environ. Microbiol. 62:4465-4470), has led to the construction of organisms capable of converting hexoses and pentoses to ethanol (cofermentation). Such microorganisms can advantageously be used in the methods of the present disclosure.

[242] In certain embodiments, the fermenting microorganism is a *Zymomonas mobilis* with improved tolerance for acetate (*see, e.g.,* U.S. Patent Publication US 2009/0221078).

[243] In certain embodiments, the fermenting microorganism is a *Zymomonas mobilis* with improved utilization of xylose (*see, e.g.,*, U.S. Patent Publication US 2009/0246846).

[244] In certain embodiment, the fermenting microorganism is a *Zymomonas mobilis* with the ability to ferment pentoses into ethanol (*see, e.g.*, U.S. Patent Publication US 2003/0162271).

[245] The improved method as described herein may result in an improved ethanol yield. The improved ethanol yield is about 0.1 to about 1.0% greater than that of an ethanol production process not featuring the glucoamylase and the added hemicellulase. The ethanol yield may be expressed as "gal UD/bushel corn," reflecting gallon of undenatured ethanol produced per bushel corn. Modern technologies typically allow for an ethanol yield in the range of about 2.5 to about 2.8 gal UD/bushel corn. *See Bothast & Schlicher,* (2005) Appl. Microbiol. Biotechnol., 67: 19-25. The improved ethanol production efficiency may attribute to more efficient starch utilization in the starch processing as described herein. At the end of ethanol production, the residual starch present in 100 gram of grain by-products is at least about 10%, about 20%, or about 30% lower than that of an ethanol production process having starch liquefied at a temperature of about 85°C and at a alpha-amylase dosage required to reach a DE value of at least about 10 within 90 minutes.

[246] In further embodiments, by use of appropriate fermenting microorganisms as known in the art, the fermentation end product may include without limitation glycerol, 1,3-propanediol, gluconate, 2-keto-D-gluconate, 2,5-diketo-D-gluconate, 2-keto-L-gulonic acid, succinic acid, lactic acid, amino acids and derivatives thereof. More specifically, when lactic acid is the desired end product, a *Lactobacillus* sp. (*L. casei*) may be used; when glycerol or 1,3-propanediol are the desired end products, *E. coli* may be used; and when 2-keto-D-gluconate, 2,5-diketo-D-gluconate, and 2-keto-L-gulonic acid are the desired end products, *Pantoea citrea* may be used as the fermenting microorganism. The above enumerated list are only examples

and one skilled in the art will be aware of a number of fermenting microorganisms that may be appropriately used to obtain a desired end product.

[247] A suitable variation on the standard batch system is the "fed-batch fermentation" system. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression likely inhibits the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors, such as pH, dissolved oxygen and the partial pressure of waste gases, such as CO_2. Batch and fed-batch fermentations are common and well known in the art.

[248] Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor, and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density, where cells are primarily in log phase growth. Continuous fermentation allows for the modulation of one or more factors that affect cell growth and/or product concentration. For example, in one embodiment, a limiting nutrient, such as the carbon source or nitrogen source, is maintained at a fixed rate and all other parameters are allowed to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, cell loss due to medium being removed should be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes, as well as techniques for maximizing the rate of product formation, are well known in the art of industrial microbiology.

[249] Optionally, following fermentation, ethanol may be extracted by, for example, distillation and optionally followed by one or more process steps. In some embodiments, the yield of ethanol produced by the present methods will be at least about 8%, at least about 10%, at least about 12%, at least about 14%, at least about 15%, at least about 16%, at least about 17%, at least about 18%, and at least about 23% v/v. The ethanol obtained according to the process of the present disclosure may be used as, for example, fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or industrial ethanol.

[250] Grain by-products from the fermentation typically are used for animal feed in either a liquid form or a dried form. If the starch is wet milled, non-starch by-products include crude protein, oil, and fiber, e.g., corn gluten meal. If the starch is dry-milled, the by-products may include animal feed co-products, such as distillers' dried grains (DDG) and distillers' dried grain

plus solubles (DDGS). When the grain is dry milled and mixed in a slurry before liquefaction and saccharification, however, no grain is left as a by-product.

IX Fermentation Media

[251] In some aspects, the SSF reactions or methods of the disclosure are performed in a fermentation medium or a complete fermentation medium. The term "fermentation medium," as used herein, refers to a medium before all of the components necessary for the SSF reaction to take place are present. A fermentation medium can thus be, for example, a medium resulting from a partial saccharification process. A fermentation medium can, in other embodiments, be a medium containing all the components necessary for the SSF reaction to take place. In that case, the fermentation medium is also termed "a complete fermentation medium." Moreover, a fermentation medium can, in yet other embodiments, be a medium wherein an SSF reaction is in progress or under way, and as such may contain certain products of saccharification.

[252] A complete fermentation medium includes enzymes capable of hydrolyzing carbohydrate-based cellulosic or other substrates, a fermenting organism, and a suitable grain-based substrate (*e.g.*, as described herein). Over the course of culturing the complete fermentation medium, fermentable sugars are formed through enzymatic hydrolysis, which are in turn metabolized by the fermenting organism to produce a fermentation product.

X. Simultaneous Saccharification and Fermentation Processes

[253] In certain aspects, an SSF reaction of the present disclosure is performed at a temperature of between 25 °C and 50 °C. For example, the SSF reaction takes place at a temperature of 25 °C or above, 28 °C or above, 30 °C or above, 32 °C or above, 35 °C or above, or 38 °C or above. For example, the SSF reaction takes place at a temperature of 50 °C or below, 45 °C or below, 40 °C or below, 38 °C or below, 35 °C or below, or 30 °C or below. For example, the SSF reaction takes place in a temperature range of from 28 °C to 45 °C, such as from 30 °C to 40 °C, from 32 °C to 38 °C. In an exemplary embodiment, the SSF reaction is carried out at a temperature of from 32 °C to 35 °C. In another embodiment, the SSF reaction is carried out at a temperature of about 32 °C. The temperature at which the SSF reaction is carried out can, for example, be adjusted up or down during the reaction.

[254] In SSF, the enzymatic hydrolysis of cellulose and the fermentation of glucose to ethanol are combined in one step (*see, e.g.,* Philippidis, 1996, Cellulose bioconversion technology, Handbook on Bioethanol: Production and Utilization, Wyman, ed., Taylor & Francis, Washington, D.C., pp. 179-212).

[255] SSF processes are usually carried out as batch fermentation processes, wherein the fermentation is conducted from start to finish in a single tank. Alternatively, SSF processes can be carried out as continuous fermentation processes which are steady-state fermentation systems that operate without interruption, and wherein each stage of the fermentation occurs in a separate section of a given fermentation system, and flow rates are set to correspond to required residence times. In other words, the individual steps in a fermentation process of the disclosure can be performed batch-wise or continuously. Processes where all steps are performed batch-wise, or processes where all steps are performed continuously, or processes where one or more steps are performed batch-wise and one or more steps are performed continuously are contemplated herein.

[256] In certain embodiments, a fed-batch SSF process may be desirable. A fed-batch process entails a batch phase and a feeding phase. The culture medium of the batch phase and the culture medium added during the feeding phase are chemically defined, and the culture medium of the feeding phase is added, at least for a fraction of the feeding phase, at a feeding rate that follows a pre-defined exponential function, thereby maintaining the specific growth rate at a pre-defined value.

[257] An SSF reaction of the present disclosure can suitably proceed for a period of 6 hours to 180 hours. For example, an SSF reaction of the disclosure can proceed for 12 hours to168 hours, 24 hours to 180 hours, 36 hours to 120 hours, 48 hours to 180 hours, 72 hours to 168 hours, 100 hours to 180 hours, etc.

[258] The SSF fermentation processes of the disclosure include, without limitation, fermentation processes used to produce fermentation products including alcohols (*e.g.*, ethanol, methanol, butanol, 1,3-propanediol); organic acids (*e.g.*, citric acid, acetic acid, itaconic acid, lactic acid, gluconic acid, gluconate, lactic acid, succinic acid, 2,5 diketo-D-gluconic acid); ketones (*e.g.*, acetone); amino acids (*e.g.*, glutamic acid); gases (*e.g.*, H_2 and CO_2), and more complex compounds, including, for example, antibiotics (*e.g.*, penicillin and tetracycline); enzymes; vitamins (*e.g.*, riboflavin, B_{12}, β-carotene); hormones, and other compounds.

[259] In certain aspects, the present disclosure provides a set of SSF conditions that are specifically suitable for use with a recombinant fermenting bacteria such as a *Zymonmonas* (*i.e.*, also termed "recombiant Zymomonas SSF conditions" herein). For example, these conditions include carrying out the SSF flask runs anaerobically under suitable recombinant *Zymomonas mobilis*, and carrying out the reaction at about 33 ºC, pH 5.8, and about 10 wt.% to 25 wt.% solids loading, dependent upon the particular substrates. These conditions also include, for example, commencing the fermentation by the addition of 10% of a suitable *Zymomonas mobilis*

strain, for example, strains ZW705 (recombinant) or ZW1 (wild-type) inoculum (5 g), into the reaction mixture without any additional nutrients.

[260] In certain aspects, the present disclosure provides a set of SSF conditions that are specifically suitable for use with a fermenting microorganism that is a fungus, for example, a *S. cerevisiae* yeast (*i.e.,* also termed "yeast SSF conditions" herein). For example, these conditions include carrying out the reaction with a suitable yeast strain, for example the THERMOSACC® DRY yeast, at 38 °C and pH 5.0, inoculation at 0.1 wt% without any additional nutrients, carrying out the SSF runs anaerobically by, for example CO_2 outgassing, using a reaction mixture comprising the liquefact, water, saccharification enzyme(s) and the yeast strain, as well as agitating the reaction vessel at an appropriate speed, for example, at 100 RPM, for a suitable period of time, for example, from 12 hours to 180 hours, such as 24 hours to 168 hours, 48 hours to 180 hours, 72 hours to 168 hours, 96 hours to 180 hours, etc.

XI. Recovery of SSF Products

[261] The fermentation product can be any substance that is produced by the fermenting organism. In a specific aspect, the substance is an alcohol. It will be understood that the term "alcohol" encompasses a substance that contains one or more hydroxyl moieties. In a specific aspect, the alcohol is arabinitol. In another aspect, the alcohol is butanol. In another aspect, the alcohol is ethanol. In another aspect, the alcohol is glycerol. In another aspect, the alcohol is methanol. In another aspect, the alcohol is 1,3-propanediol. In yet another aspect, the alcohol is sorbitol. In another more aspect, the alcohol is xylitol. *See, e.g., Gong et al.,* (1999) Ethanol production from renewable resources, in Advances in Biochemical Engineering/Biotechnology, Scheper, ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; *Silveira and Jonas* (2002) Appl. Microbiol. Biotechnol. 59: 400-408; *Nigam, and Singh,* (1995) Process Biochem. 30 (2): 117-124; *Ezeji et al.,* (2003) World J. Microbiol. Biotechnol. 19 (6): 595-603.

[262] Distillation can be performed on the fermentation broth from the fermentation step to recover the fermentation products such as, for example, ethanol. The fermentation and distillation steps can be carried out simultaneously or separately/sequentially. In some aspects, after distillation, two products are recovered: an alcohol, such as, for example, ethanol, and a fermentation rest or residual product (whole stillage). The alcohol, being an azeotropic mixture with water, is further purified in the separation step by a standard process such as, for example, molecular sieving. For example, ethanol with a purity of up to about 96 vol.% can be obtained, which can be used as, for example, fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or industrial ethanol.

[263] For other substances or fermentation products, any method known in the art can be used for recovery, including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), SDS-PAGE, distillation, or extraction.

[264] These and other aspects and embodiments of the present methods and variant cells will be apparent to the skilled person in view of the foregoing description and appended examples.

EXAMPLES

Example 1. Ethanol production in Samples with added Hemicellulases

A. Preparation of corn mash.

[265] A corn-based starch slurry (liquefact) was generated under industry standard conditions. Ground corns were slurried to obtain about 32% dry solid (DS), and the slurry pH was adjusted to about pH 5.8, and an alpha amylase (e.g., Spezyme® XTRA, Danisco US Inc) suitable for use at this pH was dosed at about 2 AAU/g DS. The slurry was then jet cooked at 107°C. The mash liquefact was held at about 85°C for about 90 minutes with an additional 2.0 AAU/g DS of alpha amylase added. The final dry solids level of the mash was about 23°C.

[266] Alternatively the corn-based starch slurry can be generated with a single enzyme dosing of 4 AAU/g DS of alpha amylase (e.g., Spezyme® XTRA, Danisco US Inc.) prior to jet cooking at the same temperature and pH as above.

B. Saccharification and Fermentation.

[267] Four fermentations of a corn mash prepared in accordance with the above were carried out in a simultaneous process with saccharification, first through a 72-hour SSF with 0.4 mg/g DS of Distillase SSF (Danisco US) and 0.1% Thermosacc dry yeast, in 400 ppm Urea, at pH 4.8, 34°C, and 100 RPM. After the 72-hour SSF, the SSF product mixtures were subject to a 0.75-hour thermal kill (e.g., boiling water bath at approximately 100°C), followed by a minor adjustment step to bring the volume of the liquefact back to the original volume by replenishing the water and other mass loss and restore the pH level using a 0.1 M sodium citrate buffer.

[268] The saccharification step was continued for another 96 hours but no further addition of yeast in order to allow the build up of glucose, this time with adding no moreglucoamylase (Distillase SSF, Danisco US Inc.) in the control sample, and adding 2.16 mg/g DS of Distillase SSF in the 2.16 DSSF sample. Furthermore, in separate samples each containing 0.4 mg/g DS of Distillase SSF, 0 mg/g of Accellerase ® TRIO®, about 1/3 of its total proteins are hemicellulases (TRIO control), 0.36 mg/g DS, and 2.16 mg/g DS were dosed. Incubation was carried out at pH 4.46-4.5, at 34°C, for about 96 hours, with gentle agitation at about 100 RPM.

[269] A Brookfield DV-E viscometer, set at Vane #73, half vane, code 79, was used to measure the viscosity of syrup at 34°C.

[270] Large increase in syrup viscosity as observed when Accellerase ® TRIO™ was dosed at 0.36 mg/g DS, but not with the DSSF sample, or with the TRIO 2.16 mg/g DS sample. This

observation corresponded to and was consistent with the reports of dramatically increased viscosity of the thin stillage and syrup in a number of corn ethanol plants when Accellerase® TRIO™ was dosed at 0.36 mg/g DS for the SSF step.

[271] The amount of fermentable sugars, glycerol and ethanol produced at different time points throughout the SSF process were determined using HPLC (Waters) and an Aminex HPX-87H column. The results of the SSF experiments are shown in **Figures 2A-2O.** As can be seen from **Figures 2A-2O,** the addition of TRIO at a comparable level as Distillase SSF resulted in an about 1% increase in ethanol yield, an about 2% increase **in glycerol yi**eld, and comparable l**evels of glucose** and xylose yields. The addition of Accellerase® TRIO™ at 0.36 mg/g DS dosing however, resulted in a loss of about 2% of ethanol yield, and about 3% of glycerol yield.

[272] All samples had comparable CO_2 production and evaporation rates.

[273] Although the foregoing compositions and methods have been described in some detail by way of illustration and examples for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be made. Therefore, the description should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

[274] All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entireties for all purposes and to the same extent as if each individual publication, patent, or patent application were specifically and individually indicated to be so incorporated by reference.

CLAIMS

What is claimed is:

1. A composition comprising a liquefied starch slurry, a glucoamylase, and one or more hemicellulases.
2. The composition of claim 1 wherein the one or more hemicellulases are present in an amount of between about 0.1 kg/metric ton and about 2.5 kg/metric ton of dry solids.
3. The composition of claim 1 or 2, wherein the starch is from corn, wheat, barley, sorghum, rye, potatoes, or any combinations thereof.
4. The composition of claim 3, wherein the starch is from corn.
5. The composition of any one of claims 1-4, further comprising one or more of enzymes selected from the group consisting of: a de-branching enzyme, a pectinase, a beta-amylase, an alpha amylase, and a phytase.
6. The composition of any one of claims 1-5, further comprising one or more of enzymes selected from the group consisting of a cellulase.
7. The composition of claim 6, wherein the cellulase is selected from a cellobiohydrolase, an endoglucanase, or a beta-glucosidase.
8. The composition of any one of claims 1-7, wherein the one or more hemicellulases are selected from a xylanase, a beta-xylosidase, or an L-alpha arabinofuranosidase.
9. The composition of claim 8, wherein at least two different hemicellulases are present.
10. The composition of claim 8, wherein at least three different hemicellulases are present.
11. The composition of claim 1, wherein the hemicellulase is present in an amount of between 0.1 kg/metric ton and about 2.5 kg/metric ton of dry solids.
12. A method of saccharifying a starch-containing substrate to prepare a fermentation feedstock comprising:
    (a) contacting a liquefied starch slurry that contains at least some cellulose and hemicellulose materials, with an enzyme mixture composition comprising a glucoamylase and one or more hemicellulases to form a substrate-enzyme mixture; and

(b) incubating the substrate-enzyme mixture for a period of time, under an incubation condition suitable for enzyme activity, to allow enzymatic reactions to occur, thereby producing a fermentation feedstock.

13. The method of claim 12, wherein the period of incubation time occur is one that is sufficient to allow (a) increase concentration of at least one fermentable sugar in the fermentation feedstock; (b) release at least one starch chain bound to or trapped by the lignocellulosic material; or (c) to hydrolyze some portion of the hemicellulosic material; wherein (a), (b) or (c) is measured compared to a control liquefied starch slurry not contacted with the one or more hemicelullases.

14. The method of claim 12 or 13, wherein the period of incubation time is between about 6 hours to about 180 hours.

15. The method of claim 14, wherein the period of incubation time is between about 24 hours to about 165 hours.

16. The method of any one of claims 12-15, wherein the substrate-enzyme mixture further comprises one or more enzymes selected from an alpha amylase, a beta-amylase, a debraching enzyme, a pectinase, or a phytase.

17. The method of any one of claims 12-16, wherein the substrate-enzyme mixture further comprises a cellulase selected from an endoglucanase, a cellobiohydrolase or a beta-glucosidase.

18. The method of any one of claims 12-17, wherein the one or more hemicellulases are selected from a xylanase, a beta-xylosidase, or an L-alpha arabinofuranosidase.

19. The method of claim 18, wherein the substrate-enzyme mixture comprises two different hemicellulases.

20. The method of claim 19, wherein the two hemicellulases are a xylanase and an arabinofuranosidase.

21. The method of claim 19, wherein the two hemicellulases are a xylanase and a beta-xylosidase.

22. The method of claim 19, wherein the two hemicellulases are a beta-xylosidase and an L-alpha arabinofuranosidase.

23. The method of claim 19, wherein the substrate-enzyme mixture comprises three different hemicellulases.

24. The method of claim 23, wherein the substrate enzyme mixture comprises a xylanase, a beta-xylosidase and an L-alpha arabinofuranosidase.

25. The method of any one of claims 12-24, wherein the incubation condition suitable for enzyme activity comprises of pH of about 3.5 to about 8.4, temperature of 30 °C to about 50 °C, and an agitation rate of 80 RPM to about 120 RPM.

26. The method of any one of claims 12-25, wherein the hemicellulases are added at about 0.1 kg/metric ton to about 2.5 kg/metric ton of dry solids in the liquefied starch slurry.

27. The method of claim 26, wherein the hemicellulases are added at about 0.5 kg/metric ton to about 1.2 kg/metric ton of dry solids in the liquefied starch slurry.

28. The method of any one of claims 12-27, further comprising fermenting the fermentation feedstock to produce a fermentation product.

29. The method of claim 28, wherein the fermentation product is ethanol.

30. The method of claim 28, wherein the yield of the fermentation product is higher by about 0.1% to about 5%, as compared to the yield of a process without the presence of the one or more hemicellulases in the substrate-enzyme mixture.

31. The method of claim 30, wherein the yield of the fermentation product is higher than about 0.2% to about 4%, as compared to the yield of a process without the presence of the one or more hemicellulases in the substrate-enzyme mixture.

32. The method of any one of claims 12-31, wherein the amount of glucoamylase is present in an amount of 0.2 mg/g to 3.0 mg/g DS.

33. The method of any one of claims 12-32, wherein the amount of cellulase is present in an amount of 0.4 mg/g to 2.6 mg/g DS.

34. The method of any one of claims 12-33, wherein the total time for incubation and fermentation steps ranges from 6 hours to 180 hours.

35. The method of claim 34, wherein the incubation and fermentation are conducted simultaneously in the same vessel.

36. The method of any one of claims 12-35, wherein the starch is from corn, wheat, barley, sorghum, rye, sweet sorghum, rice, rye, potatoes, or other suitable grains.

37. The method of claim 36, wherein the starch is corn.

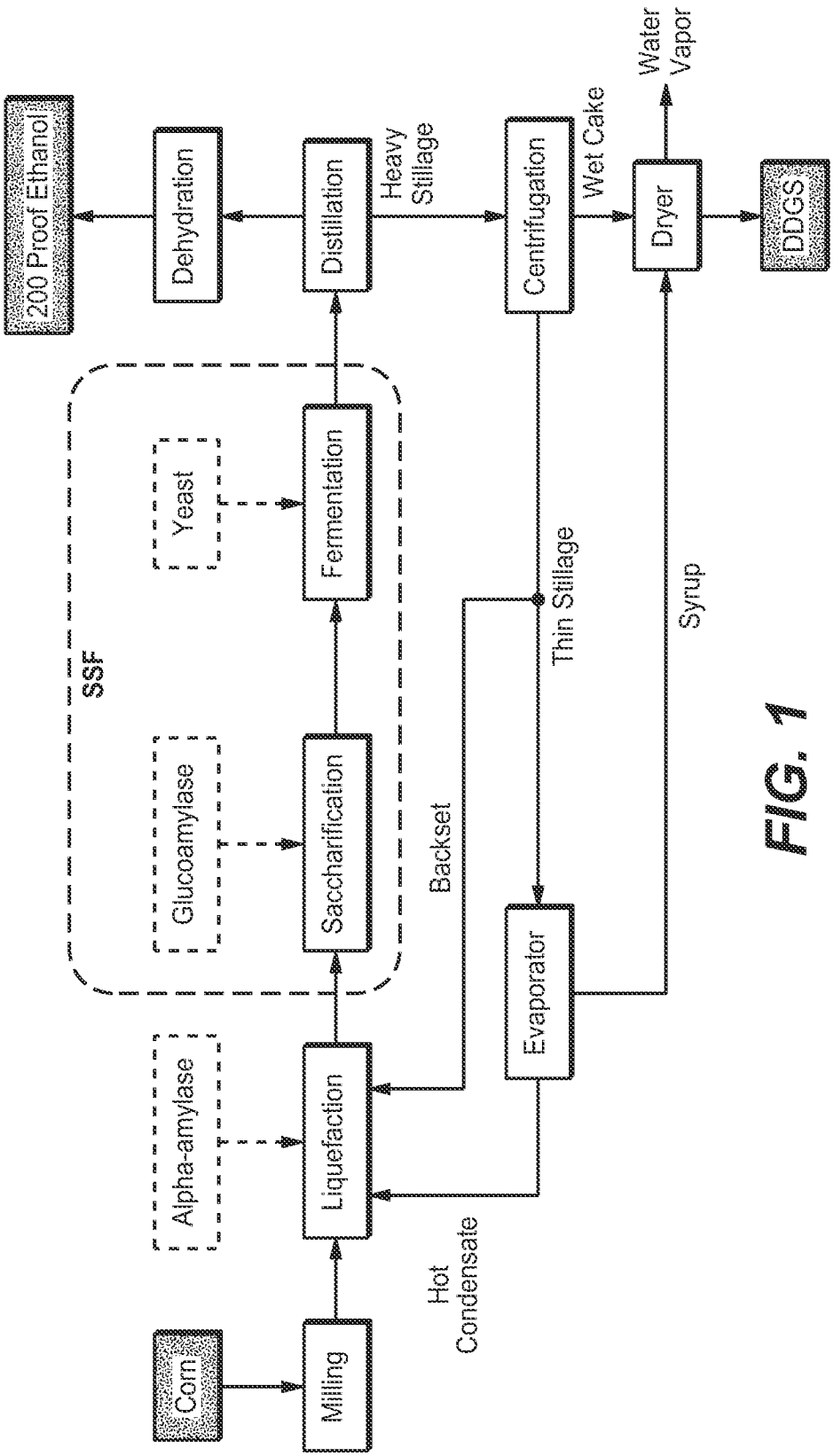
Corn
Milling
Alpha-amylase
Liquefaction
Hot Condensate
SSF
Glucoamylase
Saccharification
Yeast
Fermentation
Backset
Evaporator
Thin Stillage
Centrifugation
Heavy Stillage
Distillation
Dehydration
200 Proof Ethanol
Wet Cake
Dryer
Water Vapor
Syrup
DDGS

FIG. 1

Production of Glucose, Xylose, Glycerol and Ethanol at 72 hour SSF, in 4 aliquots of the same sample – prior to addition of Accellerase® TRIO™, volume normalized with sodium citrate buffer

** Note: markings on the X-axis indicate the amount of enzyme samples to be added in the second, continued SSF stage*

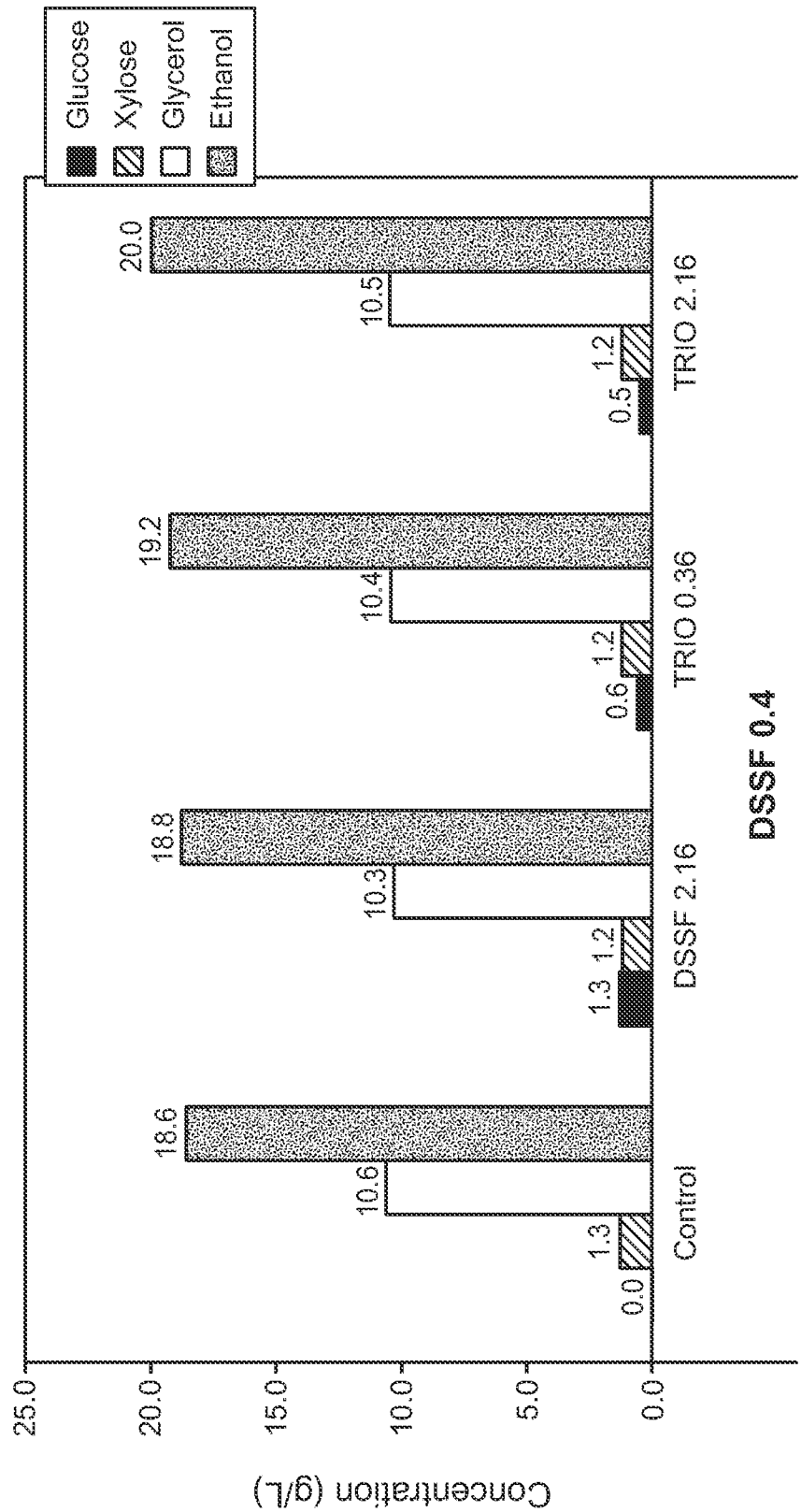

FIG. 2A

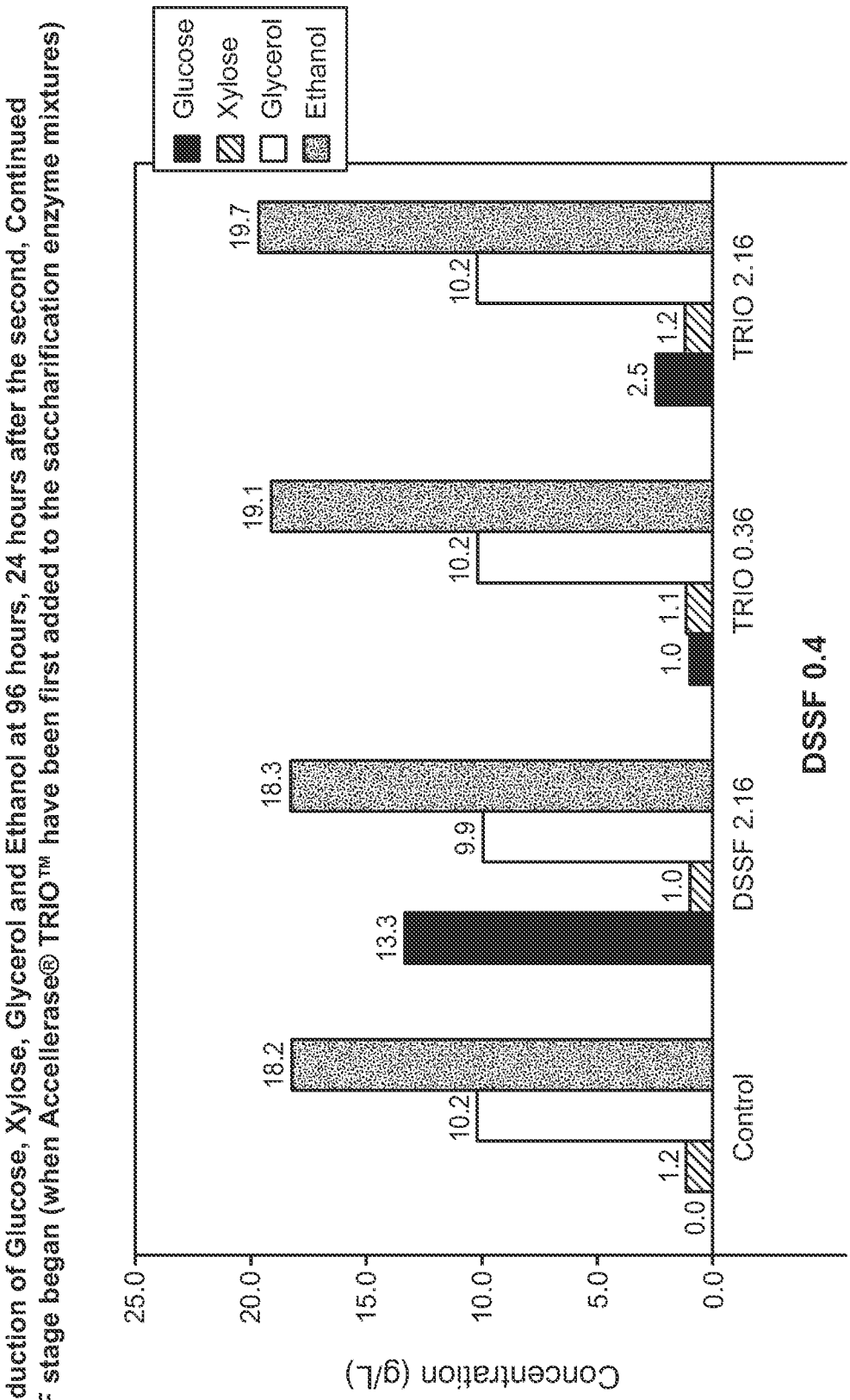
Production of Glucose, Xylose, Glycerol and Ethanol at 96 hours, 24 hours after the second, Continued SSF stage began (when Accellerase® TRIO™ have been first added to the saccharification enzyme mixtures)
Glucose
Xylose
Glycerol
Ethanol
Concentration (g/L)
25.0
20.0
15.0
10.0
5.0
0.0
0.0
1.2
10.2
18.2
13.3
1.0
9.9
18.3
1.0
1.1
10.2
19.1
2.5
1.2
10.2
19.7
Control
DSSF 2.16
TRIO 0.36
TRIO 2.16
DSSF 0.4

FIG. 2B

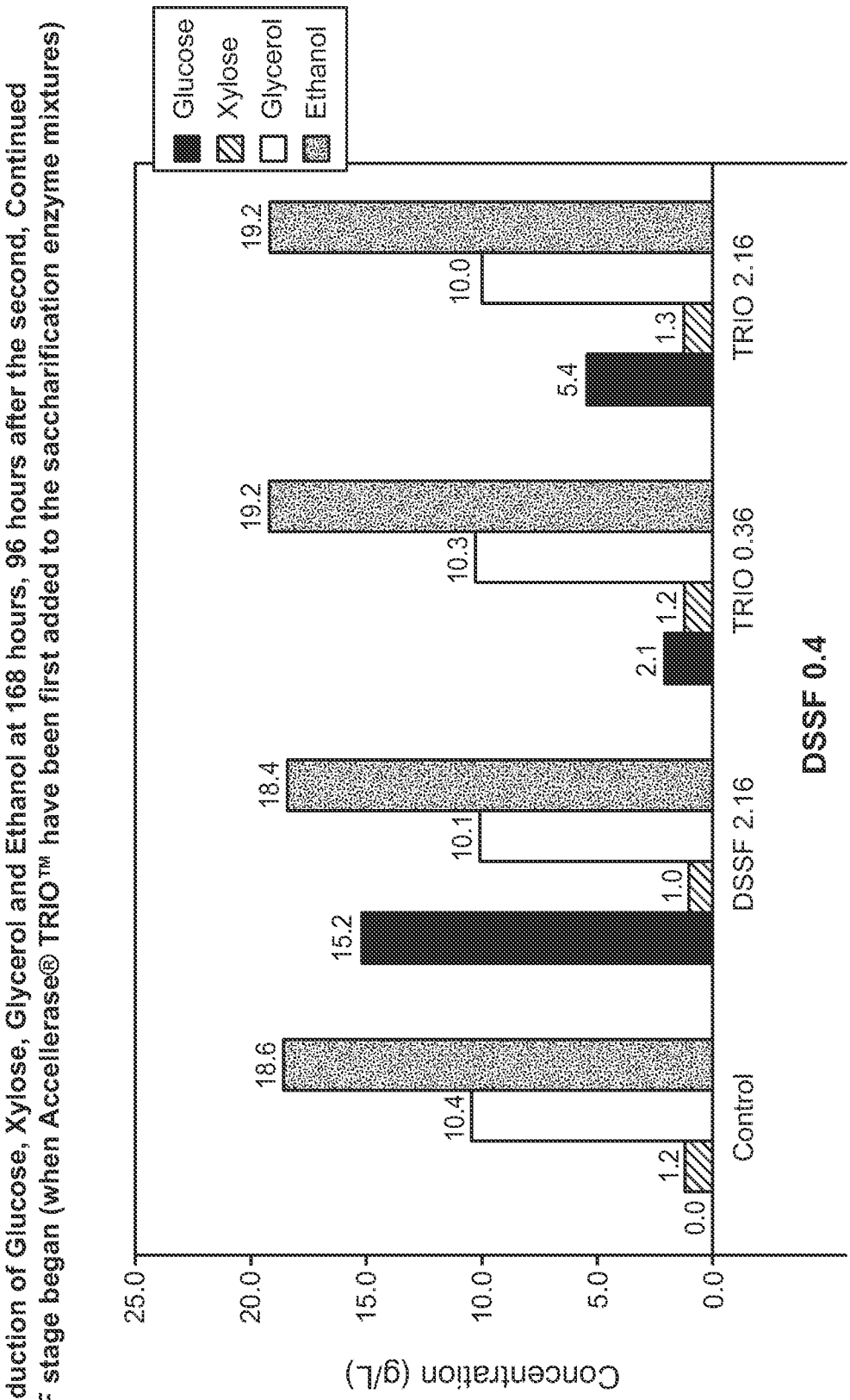
Production of Glucose, Xylose, Glycerol and Ethanol at 168 hours, 96 hours after the second, Continued SSF stage began (when Accellerase® TRIO™ have been first added to the saccharification enzyme mixtures)
Glucose
Xylose
Glycerol
Ethanol
Concentration (g/L)
25.0
20.0
15.0
10.0
5.0
0.0
0.0
1.2
10.4
18.6
1.0
10.1
15.2
1.2
10.3
18.4
2.1
1.3
10.0
19.2
5.4
19.2
Control
DSSF 2.16
TRIO 0.36
TRIO 2.16
DSSF 0.4

FIG. 2C

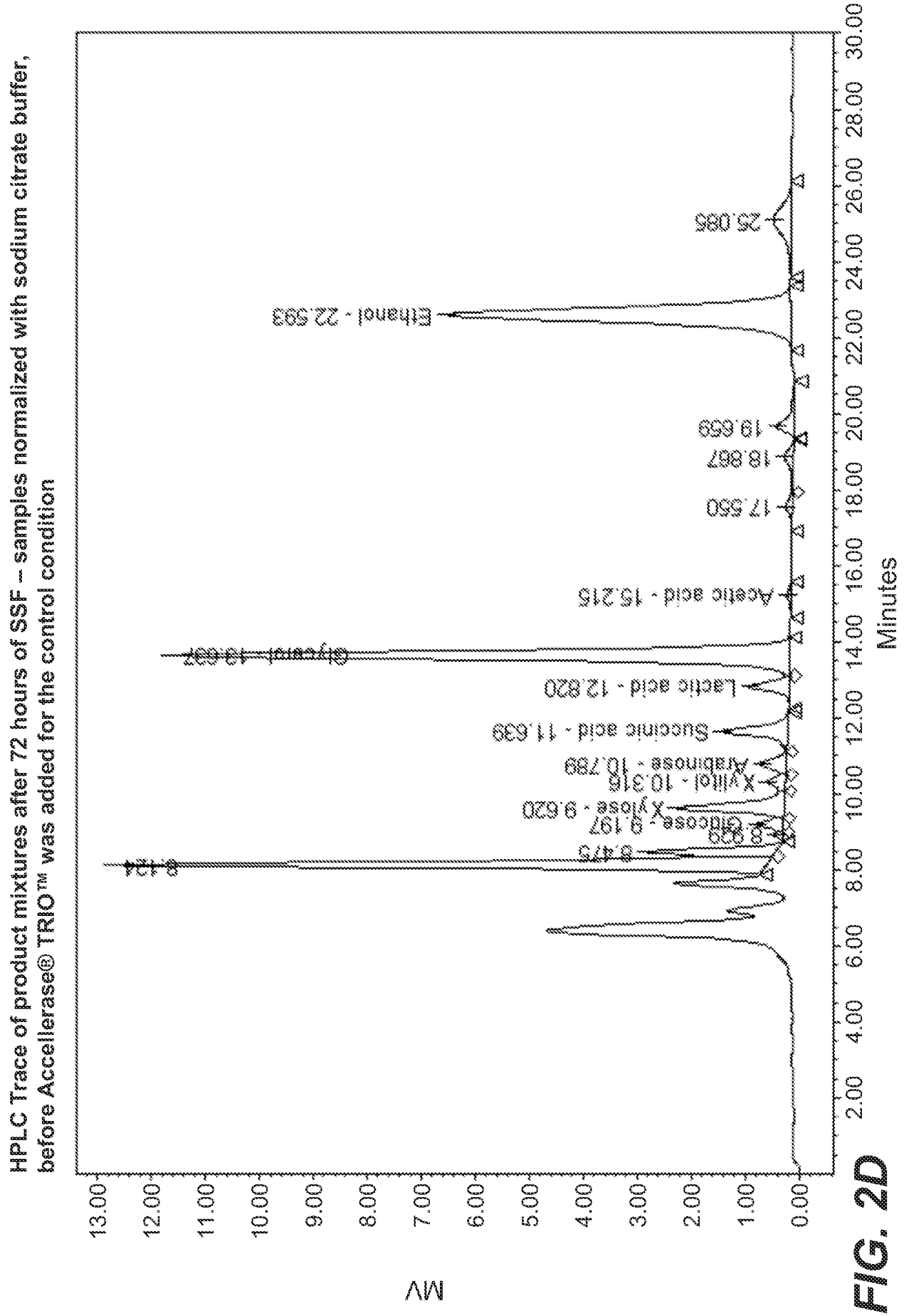
HPLC Trace of product mixtures after 72 hours of SSF – samples normalized with sodium citrate buffer, before Accellerase® TRIO™ was added for the control condition
mV
Minutes
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
Glycerol - 13.037
Lactic acid - 12.820
Succinic acid - 11.639
Arabinose - 10.789
Xylitol - 10.316
Xylose - 9.620
Glucose - 9.197
8.929
8.475
Acetic acid - 15.215
17.550
18.867
19.659
Ethanol - 22.593
25.085

FIG. 2D

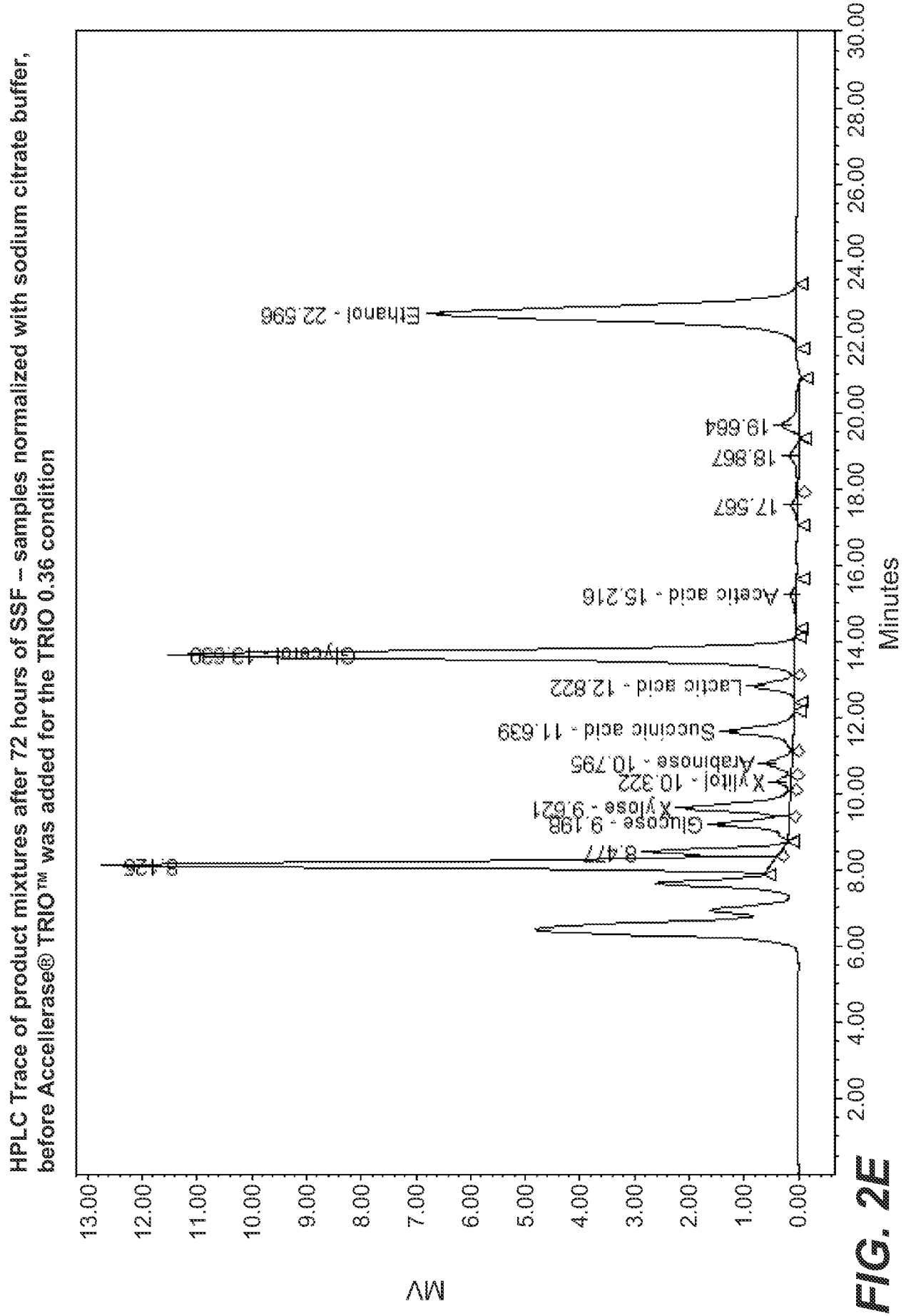
HPLC Trace of product mixtures after 72 hours of SSF – samples normalized with sodium citrate buffer, before Accellerase® TRIO™ was added for the TRIO 0.36 condition
mV
Minutes
Ethanol - 22.596
19.664
18.867
17.567
Acetic acid - 15.216
Glycerol - 13.939
Lactic acid - 12.822
Succinic acid - 11.639
Arabinose - 10.795
Xylitol - 10.322
Xylose - 9.621
Glucose - 9.198
8.477
8.125

FIG. 2E

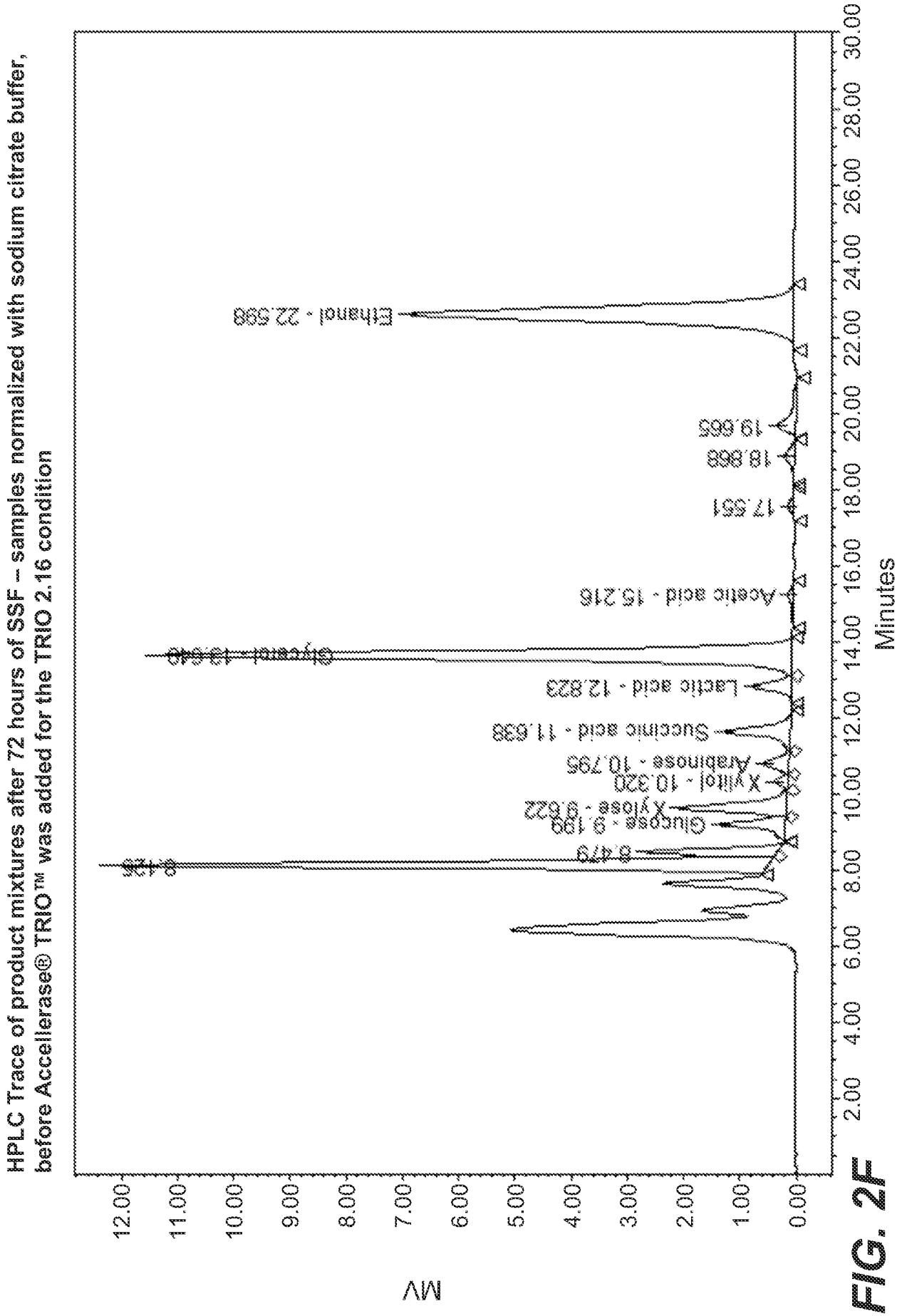
HPLC Trace of product mixtures after 72 hours of SSF – samples normalized with sodium citrate buffer, before Accellerase® TRIO™ was added for the TRIO 2.16 condition
MV
Minutes
Ethanol - 22.598
19.665
18.868
17.551
Acetic acid - 15.216
Glycerol - 13.649
Lactic acid - 12.823
Succinic acid - 11.638
Arabinose - 10.795
Xylitol - 10.320
Xylose - 9.622
Glucose - 9.199
8.479

FIG. 2F

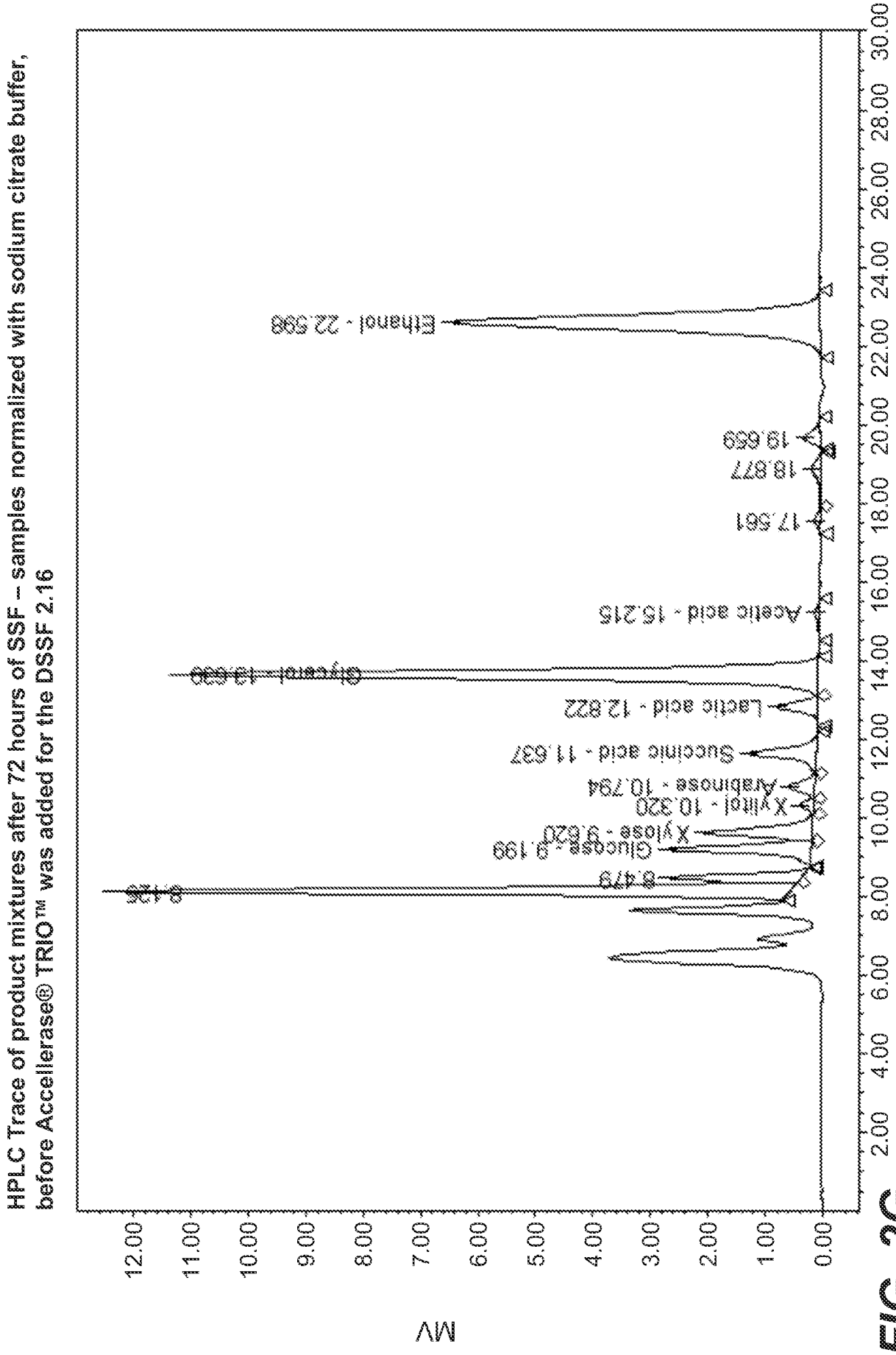
HPLC Trace of product mixtures after 72 hours of SSF – samples normalized with sodium citrate buffer, before Accellerase® TRIO™ was added for the DSSF 2.16
mV
Minutes
Ethanol - 22.598
19.659
18.877
17.561
Acetic acid - 15.215
Glycerol - 13.039
Lactic acid - 12.822
Succinic acid - 11.637
Arabinose - 10.794
Xylitol - 10.320
Xylose - 9.620
Glucose - 9.199
8.479

FIG. 2G

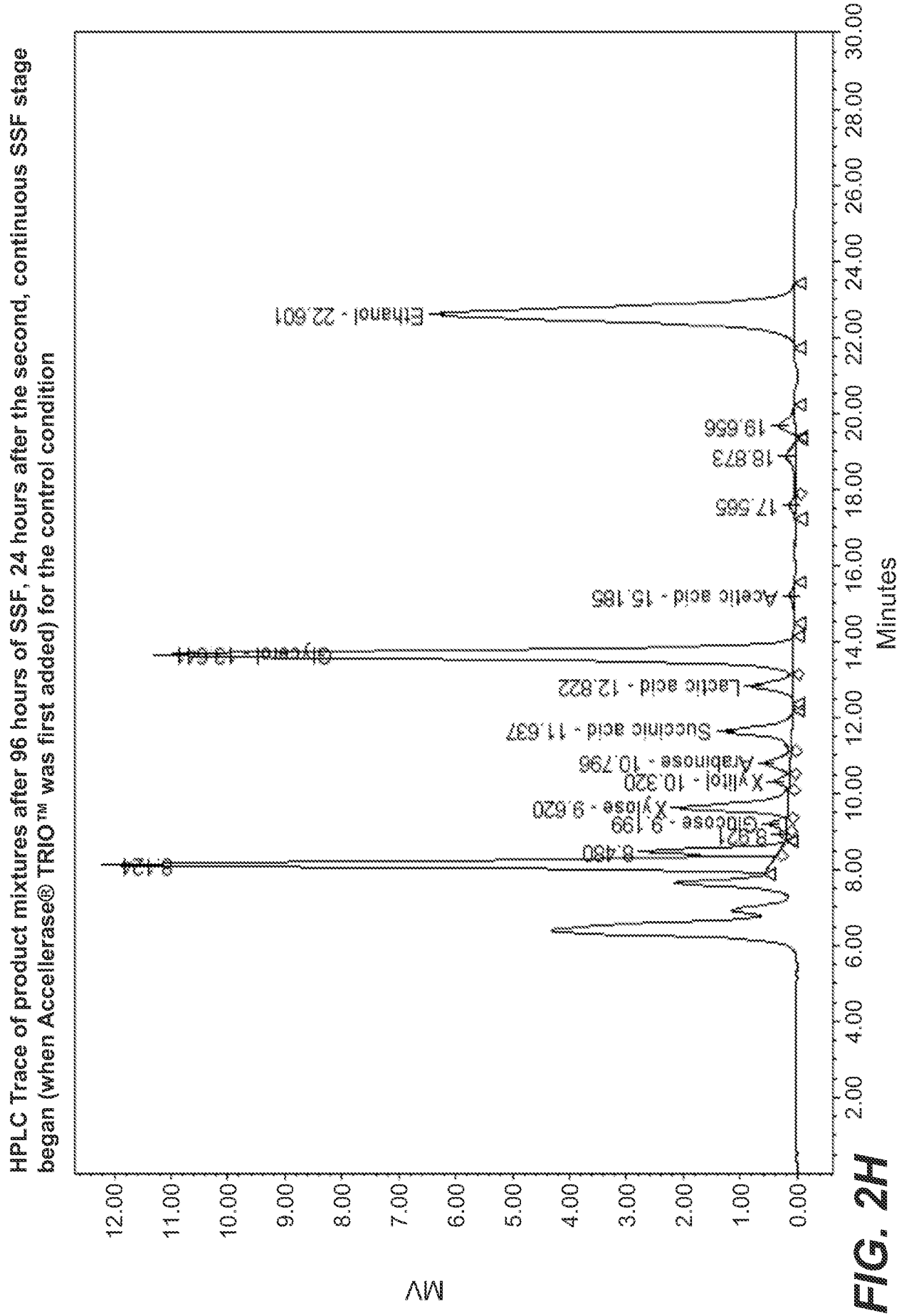
HPLC Trace of product mixtures after 96 hours of SSF, 24 hours after the second, continuous SSF stage began (when Accellerase® TRIO™ was first added) for the control condition
mV
Minutes
Ethanol - 22.601
19.656
18.873
17.565
Acetic acid - 15.185
Glycerol - 13.044
Lactic acid - 12.822
Succinic acid - 11.637
Arabinose - 10.796
Xylitol - 10.320
Xylose - 9.620
Glucose - 9.199
8.480

FIG. 2H

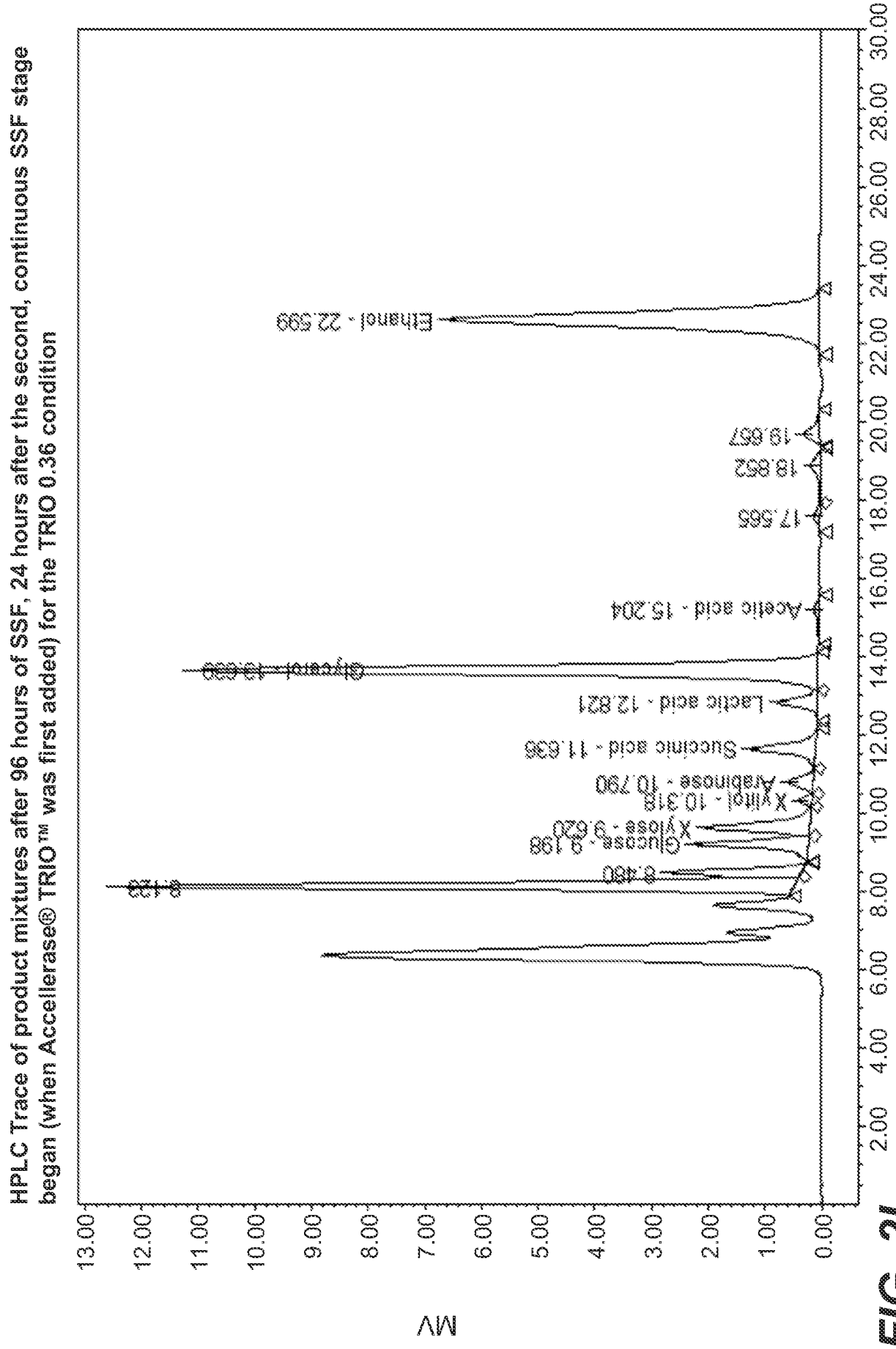
HPLC Trace of product mixtures after 96 hours of SSF, 24 hours after the second, continuous SSF stage began (when Accellerase® TRIO™ was first added) for the TRIO 0.36 condition
Ethanol - 22.599
19.057
18.852
17.585
Acetic acid - 15.204
Glycerol - 13.668
Lactic acid - 12.821
Succinic acid - 11.636
Arabinose - 10.790
Xylitol - 10.318
Xylose - 9.620
Glucose - 9.198
8.480
MV
Minutes

FIG. 2I

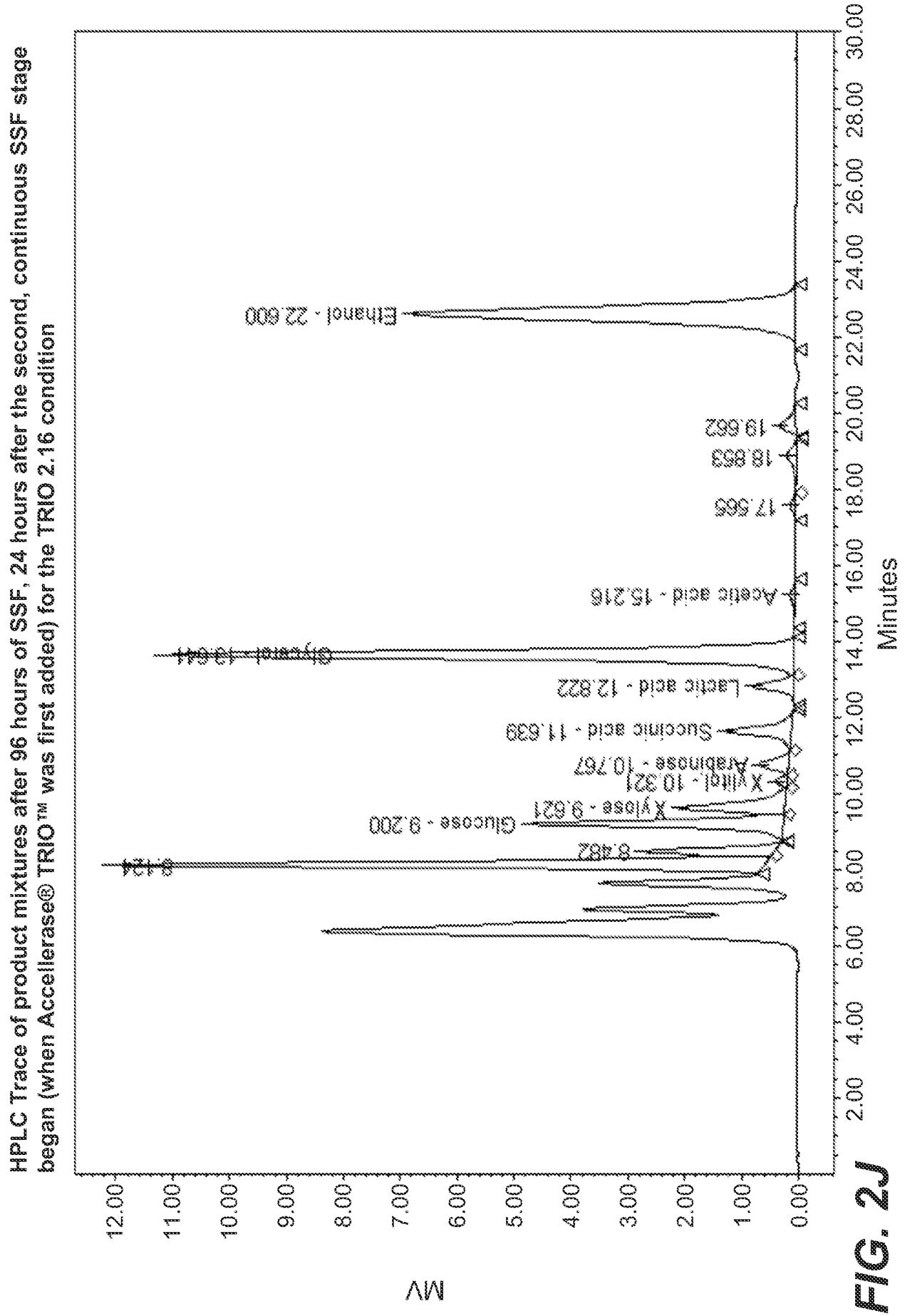
HPLC Trace of product mixtures after 96 hours of SSF, 24 hours after the second, continuous SSF stage began (when Accellerase® TRIO™ was first added) for the TRIO 2.16 condition
Ethanol - 22.600
19.662
18.853
17.565
Acetic acid - 15.216
Glycerol - 13.044
Lactic acid - 12.822
Succinic acid - 11.639
Arabinose - 10.767
Xylitol - 10.321
Xylose - 9.621
Glucose - 9.200
8.482
9.124
MV
Minutes
12.00
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00

FIG. 2J

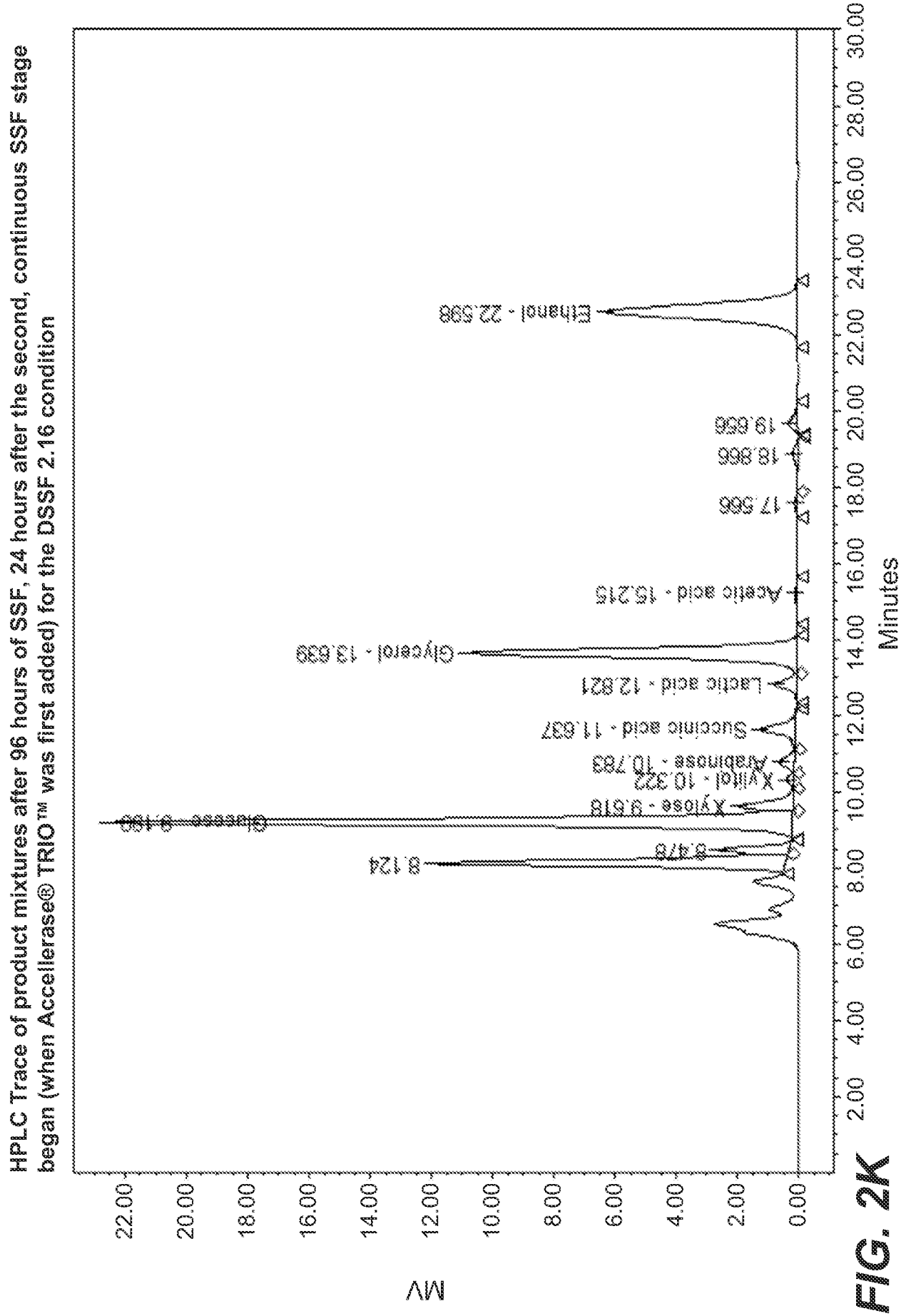
HPLC Trace of product mixtures after 96 hours of SSF, 24 hours after the second, continuous SSF stage began (when Accellerase® TRIO™ was first added) for the DSSF 2.16 condition
Glucose - 9.198
8.124
8.478
Xylose - 9.618
Xylitol - 10.322
Arabinose - 10.783
Succinic acid - 11.637
Lactic acid - 12.821
Glycerol - 13.639
Acetic acid - 15.215
17.566
18.866
19.656
Ethanol - 22.598
MV
Minutes

FIG. 2K

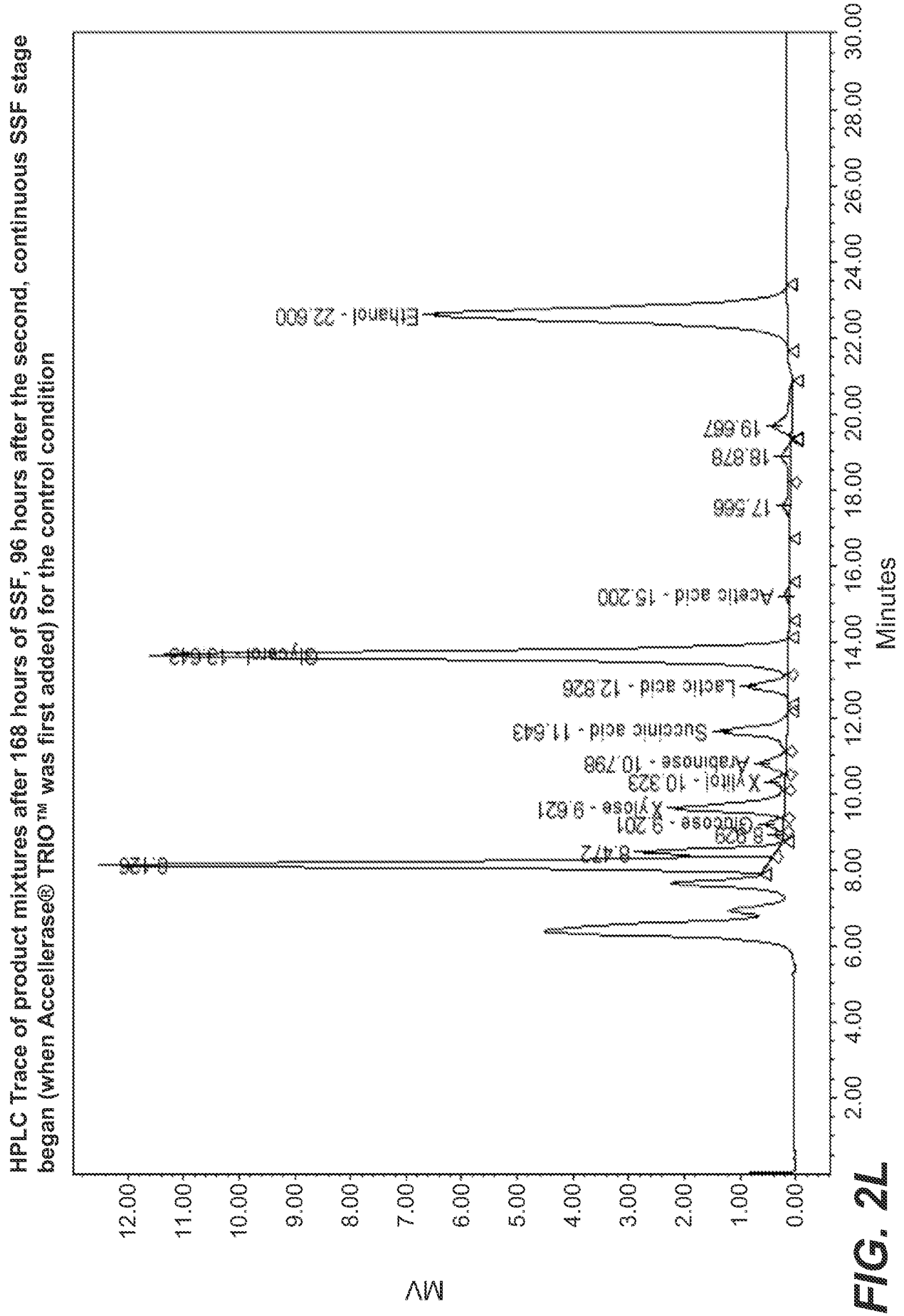
HPLC Trace of product mixtures after 168 hours of SSF, 96 hours after the second, continuous SSF stage began (when Accellerase® TRIO™ was first added) for the control condition
MV
Minutes
Ethanol - 22.600
19.667
18.878
17.566
Acetic acid - 15.200
Glycerol - 13.643
Lactic acid - 12.826
Succinic acid - 11.643
Xylitol - 10.323
Arabinose - 10.798
Xylose - 9.621
Glucose - 9.201
8.472

FIG. 2L

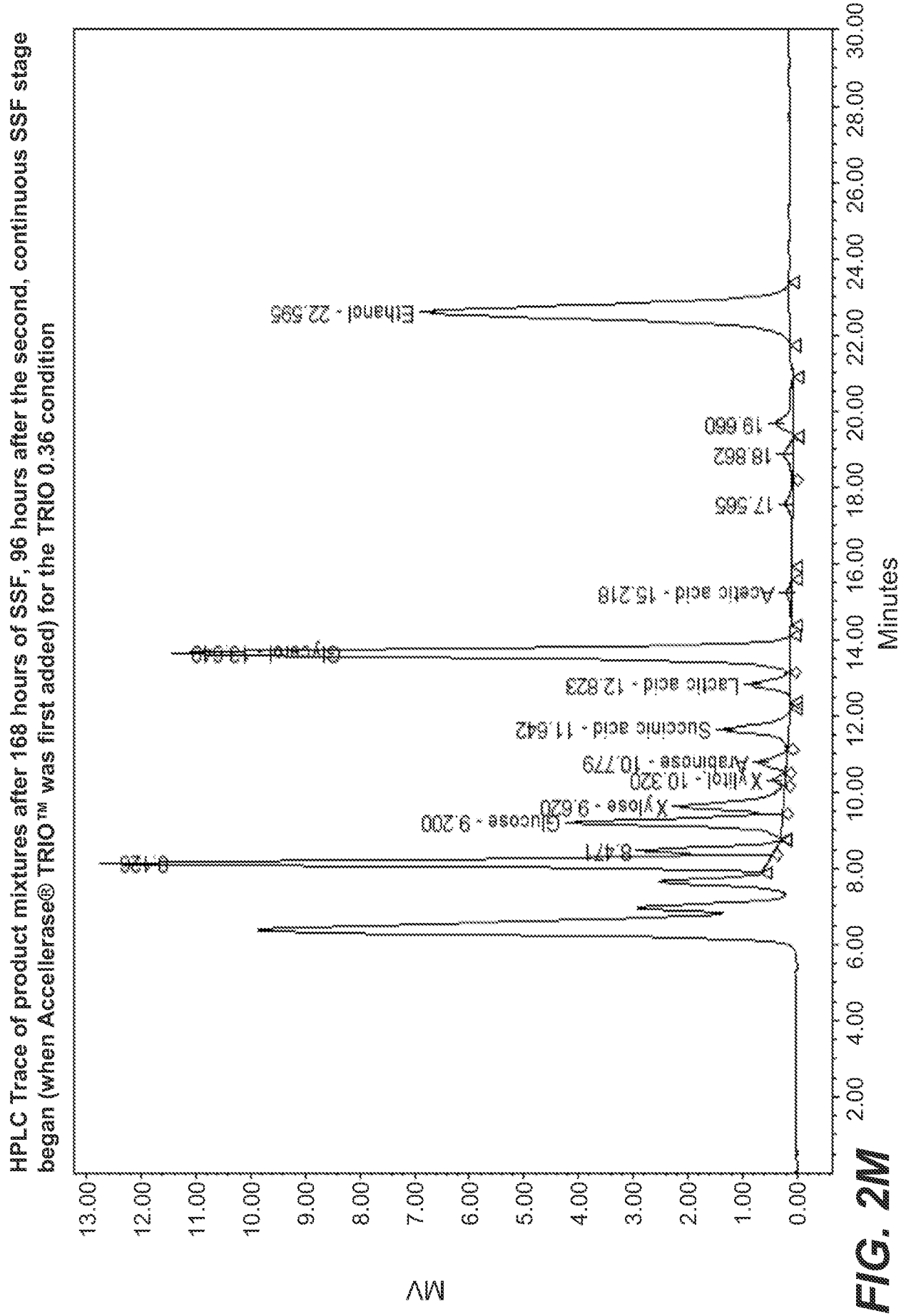
HPLC Trace of product mixtures after 168 hours of SSF, 96 hours after the second, continuous SSF stage began (when Accellerase® TRIO™ was first added) for the TRIO 0.36 condition
Ethanol - 22.585
19.660
18.862
17.565
Acetic acid - 15.218
Glycerol - 13.949
Lactic acid - 12.823
Succinic acid - 11.642
Arabinose - 10.779
Xylitol - 10.320
Xylose - 9.620
Glucose - 9.200
8.471
Minutes
MV

FIG. 2M

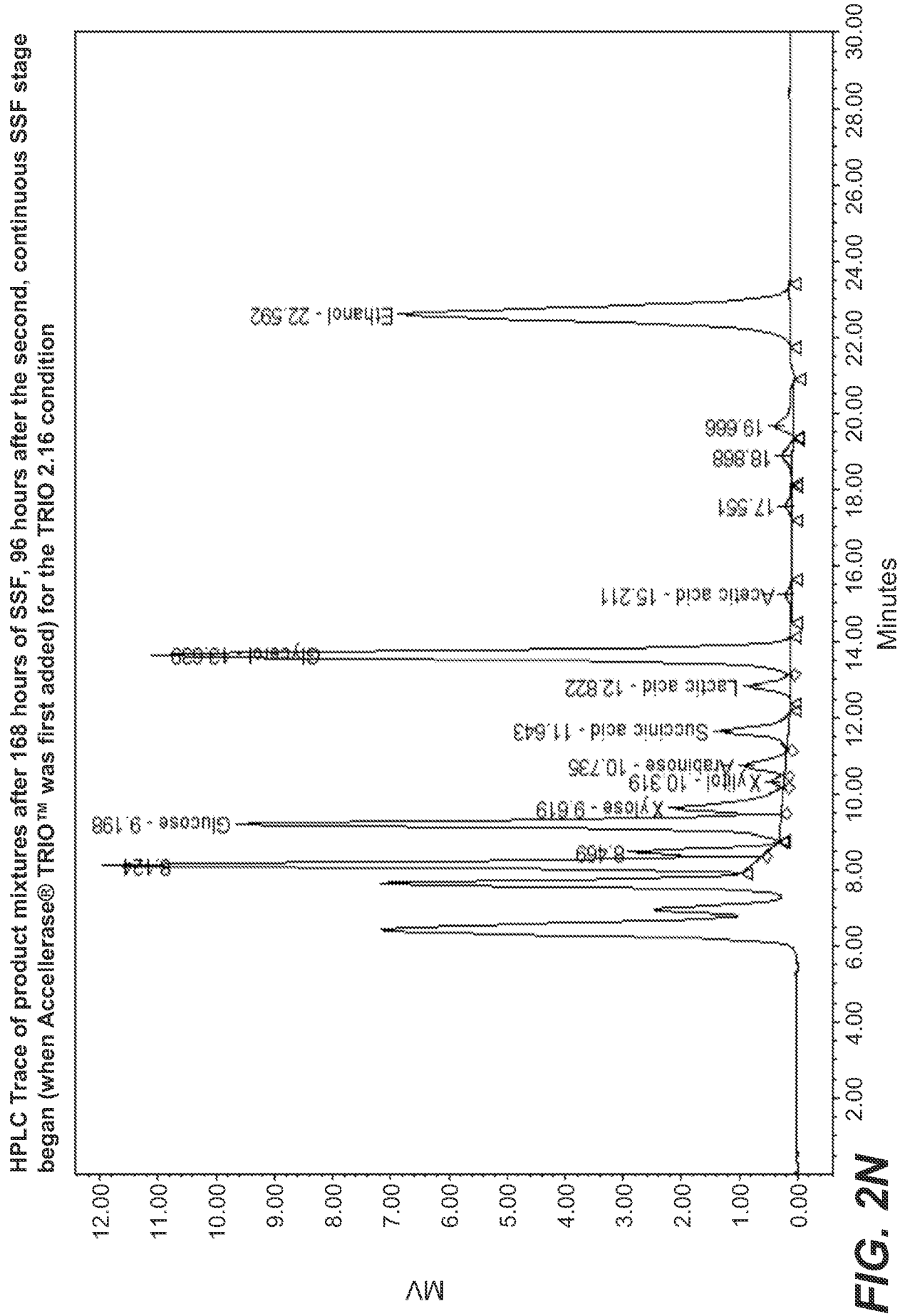
HPLC Trace of product mixtures after 168 hours of SSF, 96 hours after the second, continuous SSF stage began (when Accellerase® TRIO™ was first added) for the TRIO 2.16 condition
MV
Minutes
Ethanol - 22.592
19.666
18.868
17.351
Acetic acid - 15.211
Glycerol - 13.629
Lactic acid - 12.822
Succinic acid - 11.643
Arabinose - 10.735
Xylitol - 10.319
Xylose - 9.619
Glucose - 9.198
8.469
8.124

FIG. 2N

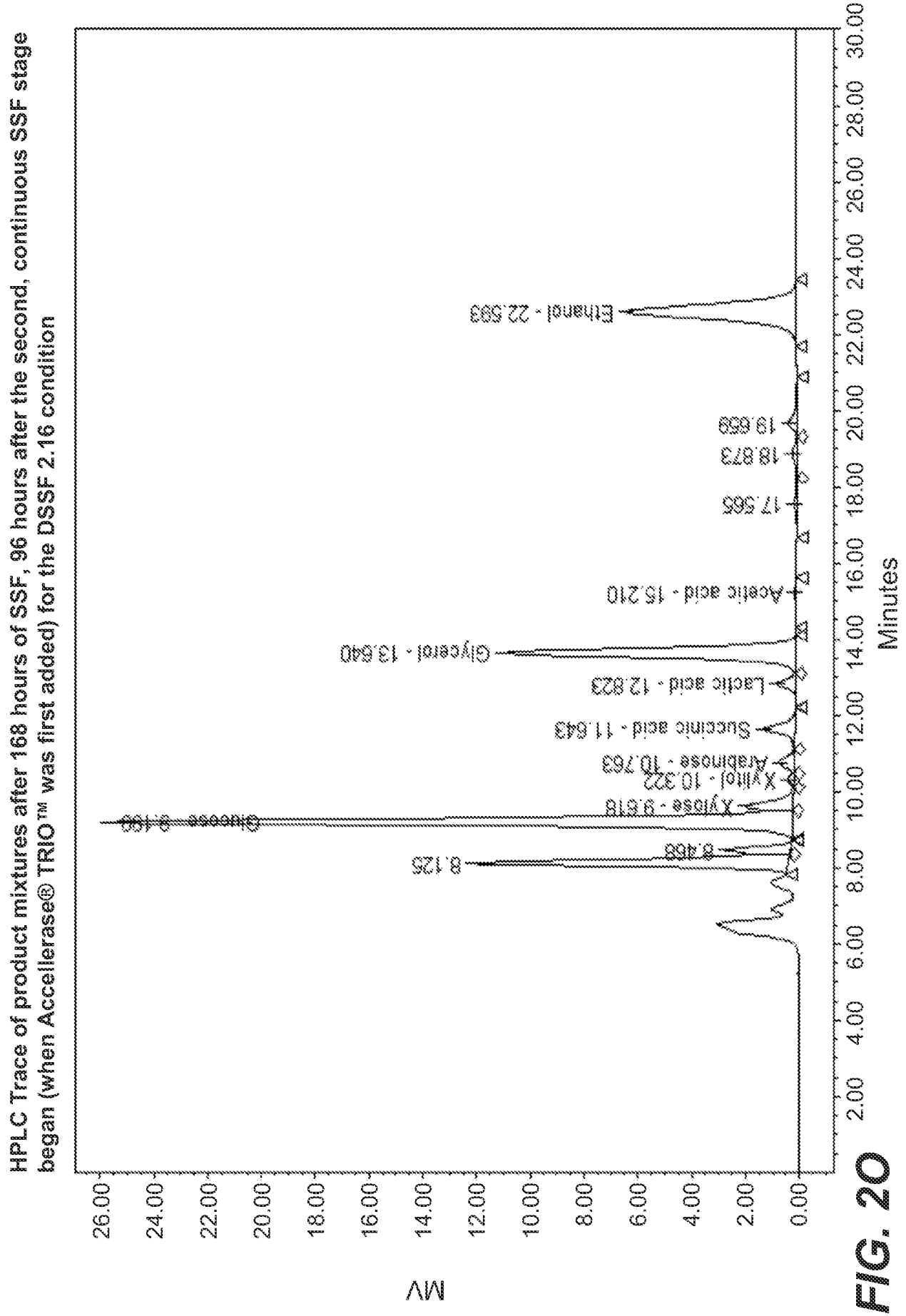
HPLC Trace of product mixtures after 168 hours of SSF, 96 hours after the second, continuous SSF stage began (when Accellerase® TRIO™ was first added) for the DSSF 2.16 condition
Ethanol - 22.583
19.659
18.873
17.565
Acetic acid - 15.210
Glycerol - 13.640
Lactic acid - 12.823
Succinic acid - 11.643
Arabinose - 10.763
Xylitol - 10.322
Xylose - 9.618
8.468
8.125
Glucose 6.150
MV
Minutes

FIG. 2O

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/060099

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/14 C12P7/06
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/149275 A1 (DANISCO US INC [US]; LI MIAN [US]; MITCHINSON COLIN [US]) 1 November 2012 (2012-11-01) claims 1-21 paragraph [0065] -----	1-37
X	WO 2009/148945 A1 (DANISCO US INC GENENCOR DIV [US]; DUAN GANG [US]; SHETTY JAYARAMA K [U) 10 December 2009 (2009-12-10) claims 1-32 ----- -/--	1,3,5-8, 12-18, 28,29, 34-36

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 2014	07/01/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Siatou, Evangelia

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/060099

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OHGREN ET AL: "A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover", PROCESS BIOCHEMISTRY, ELSEVIER, NL, vol. 42, no. 5, 5 April 2007 (2007-04-05), pages 834-839, XP022021995, ISSN: 1359-5113, DOI: 10.1016/J.PROCBIO.2007.02.003 abstract page 835, right-hand column, paragraph 2.4. page 838; table 3 -----	1-37
A	WO 2012/084225 A1 (DIREVO IND BIOTECHNOLOGY GMBH [DE]; MILOS KLAUDIJA [DE]; KOEHLER STEFF) 28 June 2012 (2012-06-28) claims 1-45 page 9, line 3 - page 10, line 26 -----	1-37

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2014/060099

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		EP 2702161 A1	05-03-2014
		JP 2014512828 A	29-05-2014
		US 2012276593 A1	01-11-2012
		WO 2012149275 A1	01-11-2012
WO 2009148945 A1	10-12-2009	AU 2009256456 A1	10-12-2009
		CA 2725737 A1	10-12-2009
		CN 102046799 A	04-05-2011
		EP 2291528 A1	09-03-2011
		US 2011124070 A1	26-05-2011
		WO 2009148945 A1	10-12-2009
WO 2012084225 A1	28-06-2012	EP 2655644 A1	30-10-2013
		US 2013330791 A1	12-12-2013
		WO 2012084225 A1	28-06-2012