



- (51) **International Patent Classification:**
C12P 7/08 (2006.01)
- (21) **International Application Number:**
PCT/GB2013/052295
- (22) **International Filing Date:**
2 September 2013 (02.09.2013)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
1215680.8 3 September 2012 (03.09.2012) GB
- (71) **Applicant:** ENSUS LIMITED [GB/GB]; The Granary,
17a High Street, Yarm, Durham TS15 9BW (GB).
- (72) **Inventor:** LYWOOD, Warwick; 7 Middleton Road, Hut-
ton Rudby, Yarm Yorkshire TS15 0JT (GB).
- (74) **Agent:** BOULT WADE TENNANT; Verulam Gardens,
70 Grays Inn Road, London Greater London WC1X 8BT
(GB).
- (81) **Designated States** (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
ZW.

- (84) **Designated States** (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- *without international search report and to be republished
upon receipt of that report (Rule 48.2(g))*
- *with sequence listing part of description (Rule 5.2(a))*

(54) **Title:** HYDROLYSIS AND FERMENTATION PROCESS

(57) **Abstract:** A method for processing the thin stillage stream resulting from a bioethanol production process comprises, firstly, partial hydrolysis of the thin stillage stream resulting from the bioethanol production process in order to convert non starch polysaccharides to soluble oligomers and monomers. Secondly, it comprises fermentation of the soluble oligomers and monomers in the partially hydrolysed thin stillage to produce ethanol. Thirdly, it comprises recycling of the product stream resulting from partial hydrolysis of the thin stillage stream and fermentation of the soluble oligomers and monomers to the front end of the bioethanol production process such that the ethanol produced during fermentation is recovered as part of a bioethanol recovery process in the bioethanol production process. A companion apparatus for processing the thin stillage stream resulting from a bioethanol production process and an animal feed product produced by the aforementioned method are also described.



WO 2014/033476 A2

HYDROLYSIS AND FERMENTATION PROCESS

FIELD OF THE INVENTION

The invention relates to methods and apparatus for bioethanol production. In particular, the invention relates to methods and corresponding apparatus which provide additional processing of the thin stillage stream resulting from a bioethanol production process. The additional processing relies upon additional hydrolysis of thin stillage and fermentation in order to produce additional ethanol. The product stream is recycled to the front end of the bioethanol production process to facilitate recovery of the additional ethanol using the existing bioethanol recovery process. The methods and apparatus may involve an additional centrifugation step to remove additional protein. This additional protein can be used as (a component of) an animal feed product or to improve the nutritional content of the animal feed products arising as part of the bioethanol production process.

BACKGROUND TO THE INVENTION

Work has been done to develop micro-organisms, including thermophilic *Geobacillus* micro-organisms, to produce bioethanol from either mixed pentose (C5) and hexose (C6) sugars, or the C5 sugars on their own. WO 2007/110606 describes thermophilic microorganisms transformed with a gene encoding an NAD-linked formate dehydrogenase in order to facilitate ethanol production. WO 2006/117536 and WO 02/29030 each describe thermophilic microorganisms carrying an inactivated lactate dehydrogenase gene.

Linde (Bioresource Technology 99 (2008) 6506 - 6511) investigated theoretical increases in ethanol yield by applying heat treatment followed by enzymatic hydrolysis to residual starch-free cellulose and hemi-cellulose fractions of slurries obtained from process streams in a starch-to-ethanol plant. The process slurries investigated were the flour, the slurry after saccharification of the starch, before fermentation, and after fermentation. An increase of 14% in ethanol yield compared with starch-only utilization could theoretically be achieved, assuming fermentation of the additional pentose and hexose sugars liberated. While cellulose hydrolysis produces glucose, which is easily fermented to ethanol, hemi-cellulose hydrolysis produces a large proportion of pentose (C5) sugars. Pentose sugars require pentose-fermenting yeast, not currently used in industrial processes.

- 2 -

DESCRIPTION OF THE INVENTION

If streams derived from stillage from a cereal based bioethanol plant are treated in order to hydrolyse some residual components, such as hemicellulose, to sugars and those sugars are fermented to ethanol, the product stream that results in a conventional plant will need to be separated into ethanol product and a residual stream. The ethanol concentration in the product stream may be relatively low and substantial cost and energy is needed to remove the ethanol. The residual product stream, following removal of ethanol also requires further processing. This additional processing is expensive and provides marginal economics for conversion of residual components in the stillage stream.

The process devised by the inventor applies to a conventional bioethanol plant (also referred to herein as a parent plant in which an existing bioethanol production process is performed). In such plants, a feedstock such as cereal is used in basic bioethanol production process steps such as milling, mashing, fermentation and ethanol recovery by distillation (discussed herein below in further detail). Following ethanol recovery, the residual whole stillage stream is separated using a centrifuge into a thin stillage stream and wet cake stream and these streams are further processed for sale. The invention relies upon taking a fraction of the bioethanol plant thin stillage stream after the centrifugation of the plant whole stillage, partially hydrolysing the stream to convert hemicellulose and cellulosic components to monomeric sugars, fermenting the sugars and other soluble non starch polysaccharides to ethanol, for example using thermophilic micro-organisms and recycling the resulting stream to the front end of the parent plant such that the bioethanol formed from the process is removed as part of the bioethanol recovery process in the parent plant. The components other than ethanol may be mixed with the whole stillage and processed for sale in the parent plant. The invention thus provides a number of advantages. More ethanol may be produced from the feedstock. Additional hydrolysis and fermentation steps can remove anti-nutritives from thin stillage and thus improve the quality of co-products. In addition, relative levels of nutritionally valuable components, in particular protein, may be increased especially where a further centrifugation step is performed as described herein.

Thus, according to a first aspect of the invention there is provided a method for processing the thin stillage stream resulting from and/or increasing ethanol yields in a (parent) bioethanol production process comprising:

- 3 -

(a) partial hydrolysis of the thin stillage stream resulting from the bioethanol production process, which partial hydrolysis converts non starch polysaccharides (found in the thin stillage) to soluble oligomers and monomers,

5 (b) fermentation of the soluble oligomers and monomers in the partially hydrolysed thin stillage to produce ethanol (additional to that produced by the bioethanol production process)

(c) recycling of the product stream resulting from steps (a) and (b) (in combination, i.e. the output of step (b)) to the front end of the bioethanol production process such that the additional ethanol produced in step (b) is recovered as part of a bioethanol
10 recovery process in the (parent) bioethanol production process.

The methods of the invention are applied to the thin stillage fraction. In a typical bioethanol production process, following distillation to remove ethanol, the resulting co-product is termed whole stillage. This whole stillage is separated, generally by decanter centrifugation, into thin
15 stillage and wet cake, with the thin stillage representing the predominantly liquid component and the wet cake representing the separated predominantly solids. The thin stillage contains potentially fermentable oligomers and monomers (sugars) that are not accessible in a standard bioethanol production process. The parent process results in fermentation of glucose derived from the starch in the feedstock. However, additional carbohydrates, such as
20 cellulose and hemicellulose, remain intact. The present invention seeks to ferment the additional sugars to produce additional bioethanol. The methods can be applied to the entire (i.e. 100% of the) thin stillage stream. In some embodiments between around 10%, 20%, 30%, 40% and around 50%, 60%, 70%, 80%, 90% or 95% of the thin stillage stream resulting from the bioethanol production process is utilised in step (a). In more specific
25 embodiments, between around 10% and around 60% of the thin stillage stream is partially hydrolysed (in step (a)). It is advantageous to utilise the thin stillage in the processes of the invention, as opposed to applying the entire thin stillage to an evaporation process to produce syrup for drying and further processing. Typically, in existing bioethanol production plants and processes, drying capacity limits the
30 throughput of the process. By diverting a proportion of the thin stillage according to the methods of the invention, to produce further ethanol from the thin stillage and facilitate bioethanol recovery, the drying capacity of the overall plant may no longer limit throughput or may do so to a lesser degree.

- 4 -

As mentioned above, in some embodiments, the thin stillage stream is produced by centrifugation of whole stillage resulting from the bioethanol production process. This centrifugation step is typically performed by decanter centrifugation. According to further preferred embodiments of the invention, the methods may further comprise a second centrifugation step applied to the thin stillage in order to remove suspended solids, predominantly protein, from the thin stillage. The second centrifugation step may utilise disk stack centrifugation in some embodiments. Disk stack centrifugation is useful in this step of the methods as it permits separation of smaller particles based upon lower solids concentrations than decanter centrifugation. Disk stack centrifugation may be used to separate protein components from the thin stillage in a continuous process based upon high centrifugal forces. In a typical disk stack centrifuge, more dense solids are forced outwards against the wall of the centrifuge and less dense liquid phases form concentric inner layers. Plates may be inserted to increase the settling surface area.

In certain embodiments, the second centrifugation step is performed at any one or more of the following stages: prior to step (a) – prior to partial hydrolysis, after step (a) but before step (b) – following partial hydrolysis but prior to fermentation; and after step (b) but before step (c) – following fermentation but prior to recycling of the product stream. It is preferred that the second centrifugation step is performed before step (a). This should maximise the efficiency of the methods as the partial hydrolysis will be performed following removal of the protein components. The protein that is removed by the additional centrifugation step is valuable, for example as an animal feed product (DG, DS, vinasse, DGS and DDGS etc.). Thus, it is generally not preferred to expose the protein component to hydrolytic conditions in step (a). It is apparent that use of the second centrifugation step is advantageous in the context of an existing bioethanol production process, for example to facilitate recovery of additional protein from the thin stillage. Accordingly, the invention also provides a bioethanol production process in which the thin stillage stream is exposed to a centrifugation step in order to deplete the thin stillage of (additional) protein. Preferably, this is achieved using disk stack centrifugation. The protein component (which may be the deposited solids that include the protein) can then be separately processed or combined into a further product such as an animal feed product. The removed solids may be used directly as a protein enriched animal feed product, optionally following drying.

- 5 -

The solids, in particular protein, removed according to the additional centrifugation step, applied to the thin stillage, can be fed into the existing bioethanol production process to upgrade the composition of downstream co-products. The removed solids, in particular protein may be added to the syrup or wet cake or may be used as, or incorporated
5 directly into, a downstream co-product. In specific embodiments, the removed solids, in particular protein are added to the wet cake prior to drying in order to increase the protein content in the wet cake. The removed solids, in particular protein may be subject to an independent drying process in certain embodiments (i.e. separate from drying of the wet cake). The dried product can then be solid as a high protein animal feed.

10 Examples of downstream co-products include distillers grain (DG), distillers dried grain (DDG), distillers solubles (DS), distillers dried grains with solubles (DDGS) and/or vinasse. The vinasse may be sugar beet vinasse. In specific embodiments, the removed protein is directed to an independent drying facility.

15 In step (c), the product stream resulting from steps (a) and (b) (in combination i.e. the stream resulting from the end of step (b)) is recycled to the front end of the bioethanol production process. This enables the additional ethanol produced by the fermentation of step (b) to be recovered as part of the bioethanol recovery process in the existing bioethanol production process. Accordingly, by "front end" is meant a point in the existing or parent bioethanol
20 production process which permits the additional bioethanol to be recovered as part of the existing or parent bioethanol production process (i.e. without requiring a separate recovery step). Thus, the product stream from steps (a) and (b) is recycled upstream of the distillation step in the existing bioethanol recovery process. This effectively means at any point up to the inlet distillation. In specific embodiments, step (c) comprises recycling of the product
25 stream resulting from steps (a) and (b) to any one or more of the process stream inlet, milling step, mashing step, liquefaction step, mash cooling step, fermentation step, the beerwell and distillation (inlet).

Describing now the (front end of the) parent or existing bioethanol production process,
30 generally, the feedstock which may be a cereal such as wheat, is quality tested prior to transfer into grain storage bins. The grain then passes (at the process stream inlet) to and through hammer mills to produce coarse particles known as "meal". This is the milling step. In the mashing step, water and enzymes (such as amylase) are added to the meal in a mixing tank to produce what is known as "mash". As part of the liquefaction step, the mash
35 is then cooked in a cooking system generally to around 100°C. This may involve use of jet-cookers that inject steam into the mash. This liquefies the starch and reduces the levels of

- 6 -

bacteria in the mash. Enzymes are then added to the liquefied mash to convert starches in the mash to simple sugars such as dextrose. In the mash cooling step, the mash is passed through a series of heat exchangers to cool the mash, to a temperature of around 30°C if yeast fermentation is to be performed or higher, such as around 50°C, if thermophilic microorganisms and in particular bacteria (as described herein) are to be used, prior to onward transmission into the fermenter. As the fermenter fills, additional enzymes (e.g. glucoamylase) may be added to complete the breakdown of starch into simple sugars (e.g. glucose). This is referred to as saccharification. In the fermentation step a suitable microorganism, such as a yeast or bacterium (discussed in greater detail herein) is added to the mash to convert sugars to ethanol (by saccharification fermentation). The process produces a "beer solution" that contains alcohol and non-fermentable solids, together with heat and carbon dioxide. The other components of the feedstock (protein, oil, etc.) remain largely unchanged during the fermentation process. In most "dry-grind" ethanol plants, the fermentation process occurs in batches. A fermentation tank is filled, and the batch ferments completely before the tank is drained and refilled with a new batch. The up-stream processes (grinding, liquefaction, and saccharification) and downstream processes (distillation and recovery) may occur continuously (grain is continuously processed through the equipment). Thus, the parent bioethanol production process may incorporate at least three fermenters (tanks for fermentation). This ensures that, at any given time, one fermenter is filling, one is fermenting (usually for at least 48 hours), and one is emptying and resetting for the next batch. Carbon dioxide is also produced during fermentation. If the carbon dioxide is not recovered it is simply released from the fermenters to the atmosphere. Preferably, the carbon dioxide is recovered. If recovered, this carbon dioxide can be compressed and sold for carbonation of soft drinks or frozen into dry ice for cold product storage and transportation. After the fermentation is complete, the fermented corn mash (now called "beer") is emptied from the fermenter into a beer well. The beer well stores the fermented beer between batches and supplies a continuous stream of material to the ethanol recovery steps, including distillation.

Thus, the parent or existing bioethanol production process includes steps of processing and fermenting a feedstock (the "front end") to produce ethanol and stillage (via distillation i.e. the bioethanol recovery process), followed by separation of whole stillage to produce thin stillage and wet cake.

The invention relies, in step (a), upon partial hydrolysis of the thin stillage stream resulting from the parent bioethanol production process, which partial hydrolysis converts non starch

- 7 -

polysaccharides (and potentially any starch that has not already been converted during the parent bioethanol production process) to soluble oligomers and monomers. The hydrolysis may partially or completely replace any backset of thin stillage which may have otherwise been employed in the parent or existing bioethanol production process. The hydrolysis is partial, which represents an important balance to ensure ethanol yields are improved compared to these achieved without hydrolysis whilst not adversely affecting the nutritional quality of the co-products of the bioethanol production process. Maximising ethanol yields by also maximising hydrolysis may be to the detriment of the quality of the downstream (animal feed) co-product. Similarly, extensive hydrolysis may increase process costs to such an extent that it becomes uneconomic. Thus, in specific embodiments the partial hydrolysis comprises up to around 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15% or around 10% hydrolysis of the non starch polysaccharides (found in the thin stillage).

The partial hydrolysis may be performed by any suitable means. In specific embodiments the partial hydrolysis is performed chemically and/or enzymatically. Typically, if chemical hydrolysis is utilised the fermentation will need to be performed separately, under different reaction conditions. In certain embodiments, particularly where enzymatic hydrolysis is performed, the partial hydrolysis and fermentation can be performed simultaneously (i.e. steps (a) and (b) would effectively be combined). Such processes may be referred to as simultaneous saccharification fermentation (SSF). Combinations of chemical and enzymatic hydrolysis may also be performed in certain embodiments.

Chemical partial hydrolysis can be performed under any suitable conditions. In certain embodiments, chemical partial hydrolysis employs an acid. In specific embodiments, the acid comprises, consists essentially of or consists of sulphuric acid or nitric acid or hydrochloric acid. Concentrated acids may be employed in suitable amounts as would be readily determined by one skilled in the art in order to achieve the desired levels of hydrolysis. In specific embodiments, the acid is employed at a concentration of around 1-10% acid, or more specifically 0.5-5% acid. Suitable acid hydrolysis conditions are described herein, in particular with reference to the experimental examples, and may be applied more generally to the invention.

Partial hydrolysis may also be performed at any suitable temperature. A temperature elevated over room (or ambient) temperature may facilitate the hydrolysis process. Thus, in specific embodiments (chemical) partial hydrolysis is performed at a temperature between

- 8 -

around 50 and 200 degrees Celsius ($^{\circ}\text{C}$), more specifically between around 100 and 150 $^{\circ}\text{C}$ and even more specifically between around 120 and 140 $^{\circ}\text{C}$. Thus, an acid may be used to hydrolyse the thin stillage at any of these temperatures.

- 5 Similarly, the partial hydrolysis is performed for an appropriate period of time to ensure the desired level of conversion of non starch polysaccharides to soluble oligomers and monomers. In certain embodiments, chemical partial hydrolysis is performed for a period of between around 10 minutes and 5 hours, more specifically between around 20 minutes and 3 hours, or even more specifically between around 30 and 120 minutes. Thus, an acid may be
10 used to hydrolyse the thin stillage over any of these time periods.

Enzyme hydrolysis may be performed instead of, or together with, chemical hydrolysis. If both approaches are combined they may be performed simultaneously, sequentially or separately. Temperature, time and concentration conditions may need to be adjusted
15 accordingly depending upon the approach taken, as would be appreciated by a skilled person. For example, enzymes may not perform efficiently at low temperatures and may be (irreversibly) denatured at higher temperatures. Thus, enzymes may be employed at a temperature of around 10 and 80 $^{\circ}\text{C}$, (the temperature of the stillage) more preferably between approximately 20 and 40 $^{\circ}\text{C}$ and often at around 37 $^{\circ}\text{C}$, unless the enzymes are
20 thermostable. The type of enzyme employed will depend upon the nature of the thin stillage, which in turn may be determined by the fermentation feedstock employed in the bioethanol production process. Typically, glycosidase enzymes are employed. In specific embodiments, enzyme hydrolysis is performed using a hemi-cellulase and/or a cellulase. Specific examples of such enzymes include glycan hydrolase, E.C.3.2.1 and/or cellulases
25 such as endo beta-glucanases and beta-glucosidase.

The parent bioethanol production process can be based upon any suitable fermentation feedstock. In specific embodiments, the process relies upon a fermentation feedstock comprising a hemi-cellulose containing material, in particular plant material. The feedstock
30 may be a cereal feedstock. Suitable examples of feedstocks include corn, wheat, barley and sugar beet pulp. The invention may rely upon thermophilic microorganisms capable of fermenting such hemi-cellulosic sugars derived from plant materials. The feedstock may additionally or alternatively comprise cellulose containing material. Fermentation may thus be of pentose and/or hexose sugars.

35

In agreement with the source fermentation feedstock, the non-starch polysaccharides may comprise, consist essentially of or consist of hemicellulose. In specific embodiments, the non starch polysaccharides comprise at least around 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% etc. hemicellulose. Similarly, in specific embodiments, the non starch polysaccharides comprise at least around 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% etc. cellulose. Cellulose and hemicellulose may make up the total of the non starch polysaccharides in certain embodiments. Other polysaccharides may be present depending upon the source of the feedstock, such as pectins, glucans, fructans, glycogen, gums and inulin and may be a source of fermentable sugars such as galactose, arabinase, xylose, glucose (D) and fructose.

As described in further detail herein, the processes and apparatuses of the invention may be specifically adapted to permit fermentation of pentose sugars. Thus, in certain embodiments, partial hydrolysis produces soluble oligomers and monomers which comprise, consist essentially of or consist of pentose sugars. The preferred microorganisms of the invention can ferment both pentose and hexose sugars and soluble oligomers and monomers will typically comprise both hexose and pentose sugars. In specific embodiments, the pentose sugars comprise, consist essentially of or consist of xylose and/or arabinose. Soluble oligomers may include disaccharides such as cellobiose and trehalose.

As described above, following partial hydrolysis of the thin stillage (step (a)), the soluble oligomers and monomers in the partially hydrolysed thin stillage are fermented to produce ethanol (step (b)). Suitable fermentation procedures are well known in the art and readily applied to optimise ethanol production. Fermentation is typically anaerobic but may be carried out under partially aerobic conditions in certain embodiments, as discussed herein. As discussed above, the fermentation may include fermentation of pentose sugars. Thus, in specific embodiments, fermentation is performed using a microorganism capable of fermenting pentose sugars, which may be a bacterium or yeast, for example. In more specific embodiments, fermentation is performed using a thermophilic microorganism, in particular a thermophilic bacterium capable of fermenting pentose sugars. The thermophilic bacterium may lack lactate dehydrogenase activity. Lactate deficient mutants have previously been shown to be capable of producing increased ethanol yields. Suitable techniques for inactivating the *ldh* gene (encoding lactate dehydrogenase) are described for example in WO 2007/110608, WO 02/29030 and WO 2006/117536, the relevant disclosure of each of which is incorporated herein in its entirety. Thus, the *ldh* gene may be inactivated through an insertion, deletion or substitution mutation. Lactate production stops and the

- 10 -

excess pyruvate diverts mainly into the growth-linked pyruvate formate lyase (PFL) pathway. Thus, the thermophilic bacteria typically express pyruvate formate lyase. However, at very high sugar concentrations and/or at acid pH, the PFL pathway flux decreases and the excess pyruvate then overflows into an anaerobic pyruvate dehydrogenate (PDH) pathway, which ultimately yields only ethanol and CO₂. Therefore the preferred conditions to obtain high ethanol yields may be those that reduce flux through the PFL pathway and increase flux via the PDH pathway (Hartley, B.S. and Shama, G. Proc. Roy. Soc. Lond. 321, 555-568 (1987)). Unfortunately, under such conditions the cells may experience metabolic stress, with reduced ATP production, and a potential imbalance in NAD/NADH and CoA/acetyl CoA ratios.

In order to address this possible issue of redox imbalance, especially under conditions of high sugar levels (produced by the partial hydrolysis), in certain embodiments, the thermophilic bacterium expresses a heterologous NAD-linked (or NAD-dependent) formate dehydrogenase (FDH). Many genes encoding NAD-linked FDH are known in the art (see for example Nanba et al (Biosci. Biotechnol. Biochem. 67(10), 2145-2153 (2003)) and may be employed to transform a suitable thermophilic bacterium. Thus, the thermophilic bacterium may be transformed with an *fdh* gene, in particular an *fdh1* gene. The thermophilic bacterium may incorporate a gene encoding a thermostable NAD-linked formate dehydrogenase in certain embodiments. In other embodiments, the thermophilic bacterium may be transformed with a gene whose nucleotide sequence has been codon optimised to facilitate expression by the thermophilic bacterium. Production of such a thermostable NAD-linked formate dehydrogenase is described in detail in WO 2007/110608, the relevant disclosure of which is incorporated herein in its entirety. In a specific embodiment, the gene encoding an NAD-linked formate dehydrogenase comprises, consists essentially of or consists of the nucleotide sequence set forth as SED ID NO: 1. In a further embodiment, the thermophilic bacterium incorporates a codon optimised (for expression in (Geo)Bacillus) gene encoding a thermostable NAD-linked formate dehydrogenase comprising, consisting essentially of or consisting of the nucleotide sequence set forth as SEQ ID NO:2. This sequence includes, in addition to the basic thermostable NAD-linked dehydrogenase sequence, promoter and terminator regions and also suitable restriction sites, such as *Xba*1 sites to facilitate cloning of the gene into a suitable DNA construct.

In a still further embodiment the gene encoding an NAD-linked formate dehydrogenase is the *fdh1* gene. The *fdh1* gene may be derived from any suitable source and is preferably codon optimised for expression in the relevant thermophilic bacterium.

- 11 -

The fermentation thus may utilise a synthetic NAD-linked formate dehydrogenase, designed for optimum gene expression due to the use of the codon preferences of the appropriate thermophilic bacterium. The synthetic gene may contain engineered restriction sites to facilitate insertion into the lactate dehydrogenase gene (in frame relative to the *ldh* promoter).

5 Thereby inactivation of the *ldh* gene and expression of the *fdh* gene are achieved in a single operation. In specific embodiments, the thermostable NAD-linked formate dehydrogenase remains functional at or above a temperature of 60°C. The thermostable enzyme may be encoded by a nucleotide sequence which has been codon optimised for expression in a thermophilic bacterium. The formate dehydrogenase may comprise, consist essentially of or
10 consist of the amino acid sequence set forth as SEQ ID NO: 3, as described in WO 2007/110608, the relevant disclosure of which is incorporated herein in its entirety. Here, a specific thermostable NAD-linked formate dehydrogenase was designed based upon the amino acid sequence of the *Pseudomonas* sp 101 formate dehydrogenase (SEQ ID NO: 3) and through use of optimised codons for *Geobacillus thermoglucosidasius*. The skilled
15 person will appreciate that derivatives of this basic sequence will retain functionality. For example, conservative and semi-conservative substitutions may result in thermostable NAD-linked formate dehydrogenases and these derivatives are intended to fall within the scope of the invention provided they retain effective catalytic activity and thermostability such that they are useful in ethanol production using thermophilic bacteria. Similarly, minor deletions and/or
20 additions of amino acids may produce derivatives retaining appropriate functionality.

In certain embodiments, in order to address the possible issue of redox imbalance, the fermentation process may be carried out under partially aerobic conditions. As the PDH pathway also operates under aerobic conditions where its operation leads to mainly cell
25 mass production, the metabolic stress mentioned above can be relieved by partial sparging of air, generally performed at an optimum air sparging rate. By optimum air sparging rate is meant a sparging rate that is (just) sufficient to relieve the metabolic stress by allowing a low level of flux through the aerobic PDH pathway. This low level of flux does not, however, allow any significant decrease in the anaerobiosis and hence in the anaerobic PDH flux of
30 the process. This means that there should be no significant decrease in ethanol production levels. Furthermore, because of the severe sensitivity of the PFL pathway towards air, this air sparging may have the additional benefit of reducing the flux through the PFL pathway and further increasing the flux through the anaerobic PDH pathway, but without putting the microorganism under metabolic stress. Suitable air sparging rates can readily be determined
35 by one skilled in the art by investigating in the context of any particular fermentation process which rates result in optimal ethanol production levels and/or which minimise or reduce

- 12 -

production of formate and acetate. Air sparging may be periodic or continuous and the rate can be adjusted accordingly. The skilled person would also realise that equivalent techniques to sparging could be employed to expose the fermentation to a limited amount of air, to achieve the desired effect. Also, the skilled person would realise that air could be replaced by an alternative oxygen source if desired and the rates altered (reduced) accordingly. Thus, in a further aspect, the invention relates to ethanol production from (C5 and C6) sugars under optimum air sparging levels. Optimisation is achieved by monitoring the redox level at which the lowest formate and acetate levels result from the fermentation, and/or at which the (comparatively or correspondingly) highest level of ethanol concentrations are achieved in the process.

Any suitable thermophilic bacterium may be employed in the methods of the invention. In specific embodiments, the thermophilic bacterium is in the family Bacillaceae; more particularly the thermophilic bacterium may be of the genus *Geobacillus*. In specific embodiments, the *Geobacillus* comprises *Geobacillus thermoglucosidasius* or *Geobacillus stearothermophilus*, in particular a strain of *Geobacillus thermoglucosidasius* or *Geobacillus stearothermophilus* transformed with a gene encoding an NAD-linked formate dehydrogenase.

Whilst thermophilic bacteria have low tolerance to ethanol, this can conveniently be overcome in the fermentation by regular or continuous removal of the product stream via step (c). This ensures that the ethanol concentration in the fermentation is kept below the ethanol tolerance of the thermophilic bacterium. Fermentation may be performed within a temperature range of around 40°C and 80°C in some embodiments, such as between around 50°C and 70°C.

The fermentation in the existing (or parent) bioethanol production process may likewise be performed using thermophilic bacteria. Here, ethanol may be continuously and conveniently removed from the (high temperature) fermentation by evaporation or distillation, such as membrane and/or mild vacuum evaporation for example.

In a standard or unmodified bioethanol production process, the thin stillage, minus any thin stillage returned to the front end as backset, would undergo evaporation to produce syrup (see figure 1). Syrup may then be combined with wet cake and dried to produce a co-product which is routinely used as animal feed Dried Distillers Grains with Solubles (DDGS). By performing the additional hydrolysis and fermentation steps on the thin stillage, according

- 13 -

to the methods of the invention, the nutritional value of the co-product may be increased. The methods of the invention may, therefore, result in production of a co-product with improved nutritional content, wherein the improved nutritional content comprises (relative to the content if steps (a) and (b) had not been performed, i.e. arising from the unmodified parent bioethanol production process) one or more of decreased levels of pentose sugars, increased protein concentration, decreased fibre concentration, decreased levels of soluble oligomers and monomers, decreased levels of reducing sugars. By adding the centrifugation step to the process, as discussed above, in order to remove additional protein (from the thin stillage), the protein content of the co-product may also be increased still further (in absolute terms). In view of these benefits afforded to the co-products of the bioethanol production process, the invention also relates in a further aspect to an animal feed product produced according to the methods of the invention. The animal feed product is nutritionally enhanced, as discussed above.

The invention also relates to an apparatus for performing the methods of the invention. Accordingly, the invention also provides an apparatus for processing the thin stillage stream resulting from and/or increasing ethanol yields in a (parent or existing) bioethanol production process comprising:

(a) a hydrolyser connectable or connected to a thin stillage stream resulting from a bioethanol production process for partial hydrolysis of the thin stillage stream, which partial hydrolysis converts non starch polysaccharides to soluble oligomers and monomers

(b) a fermenter connectable or connected to the hydrolyser for fermentation of the soluble oligomers and monomers in the partially hydrolysed thin stillage to produce ethanol (additional to that produced by the bioethanol production process)

(c) a recycler connectable or connected to the fermenter for recycling of the product stream resulting from steps (a) and (b) to the front end of the bioethanol production process such that the additional ethanol produced in step (b) is recovered as part of a bioethanol recovery process in the bioethanol production process.

The hydrolyser and fermenter may comprise large stirred vessels. The vessels typically include an inlet and outlet. The recycler may incorporate suitable pipe work which connects to the outlet of the fermenter and also connects to one or more locations at the front end of the bioethanol production process, as defined herein.

In certain embodiments, the apparatus further comprises:

- 14 -

(d) a centrifuge for removal of suspended protein from thin stillage and which is connectable or connected to the hydrolyser or the fermenter.

The centrifuge may be a disk stack centrifuge as discussed above. The apparatus may also comprise means for combining the protein recovered from the centrifuge with the wet cake, syrup or combined (mixed) product for drying and inclusion in a co-product. The apparatus may also comprise means for recovering the protein from the centrifuge and which delivers the protein directly as a product or to the co-product and which is connectable or connected to the centrifuge. This may comprise a suitable mixing vessel for example where the protein can be mixed with wet cake, for example following drying, to produce the DDGS.

In some embodiments, the recycler is connected downstream of the centrifuge, which in turn is connected downstream of the fermenter such that the thin stillage stream, following hydrolysis at the hydrolyser, passes from the fermenter to the centrifuge and the liquid stream from the centrifuge passes to the recycler following centrifugation.

However, in preferred embodiments, the centrifuge is connected upstream of the hydrolyser and thus removes suspended protein from the thin stillage stream prior to partial hydrolysis of the resultant stream. This is advantageous because it permits removal of the valuable protein component prior to hydrolysis.

In some embodiments, the centrifuge is connected downstream of the hydrolyser and thus removes suspended protein from the thin stillage stream following partial hydrolysis of the thin stillage stream.

In still further embodiments, the centrifuge is connected upstream of the fermenter such that the thin stillage stream passes from the hydrolyser to the centrifuge and the liquid stream from the centrifuge passes to the fermenter following centrifugation.

The recycler is connectable or connected to the bioethanol production process upstream of the distillation unit or units, to enable the existing distilling capabilities to be utilised. The recycler may be connectable or connected to the front end of the bioethanol production process at any one or more of the process stream inlet, the miller, the masher, the liquefier, the mash cooler, the fermenter or the fermentation inlet, the beerwell and the distillation inlet.

The recycler may connect into one or more the inlets or existing pipe work between the components of the bioethanol production process. Additionally or alternatively, the recycler

- 15 -

may connect directly into one or more of the components (e.g. fermentation vessel) of the bioethanol production process.

5 By "connected" is meant a functional or operational link between the respective apparatus elements to permit the processes of the invention to be performed. Thus, for example, a connection between the hydrolyser and the thin stillage stream allows the thin stillage stream to enter the hydrolyser. Suitable connections are shown schematically in the figures.

10 As indicated in respect of the methods of the invention, which discussion and embodiments apply *mutatis mutandis* to the apparatus of the invention, partial hydrolysis of the thin stillage stream converts (non starch) polysaccharides in the thin stillage to soluble oligomers and monomers. The hydrolysis is partial, which represents an important balance to ensure ethanol yields are improved compared to these achieved without hydrolysis whilst not adversely affecting the nutritional quality of the co-products of the bioethanol production
15 process.

The partial hydrolysis may be performed by any suitable means. In specific embodiments the partial hydrolysis is performed chemically and/or enzymatically. Typically, if chemical hydrolysis is utilised the fermentation will need to be performed separately, under different
20 reaction conditions. In certain embodiments, particularly where enzymatic hydrolysis is performed, the partial hydrolysis and fermentation can be performed simultaneously (i.e. steps (a) and (b) would effectively be combined). Such processes may be referred to as simultaneous saccharification fermentation (SSF). Combinations of chemical and enzymatic hydrolysis may also be performed in certain embodiments. In such embodiments, the
25 fermenter and hydrolyser components of the apparatus may be combined.

Chemical partial hydrolysis can be performed under any suitable conditions. In use, the hydrolyser may contain an acid. In specific embodiments, the acid comprises, consists essentially of or consists of sulphuric acid or nitric acid or hydrochloric acid. Concentrated
30 acids may be employed in suitable amounts as would be readily determined by one skilled in the art in order to achieve the desired levels of hydrolysis. In specific embodiments, the acid is employed at a concentration of around 1-10% acid, or more specifically 0.5-5% acid. Suitable acid hydrolysis conditions are described herein.

35 Partial hydrolysis may also be performed at any suitable temperature. A temperature elevated over room (or ambient) temperature may facilitate the hydrolysis process. Thus, in

- 16 -

specific embodiments the hydrolyser contains a heating element to achieve a temperature of (at least) between around 50 and 200 degrees Celsius (°C), more specifically (at least) between around 100 and 150°C and even more specifically between around 120 and 140°C. Thus, an acid may be used to hydrolyse the thin stillage at any of these temperatures. The hydrolyser may comprise a suitable control system, and may incorporate a thermostat, to sense and control the temperature in the hydrolyser and/or a separate heating vessel to heat the acid to the appropriate temperature.

Similarly, the partial hydrolysis is performed for an appropriate period of time to ensure the desired level of conversion of non starch polysaccharides to soluble oligomers and monomers. In certain embodiments, the apparatus further comprises sensors and/or a suitable control system (which may be combined with a temperature control system) to permit partial hydrolysis to proceed for a period of between around 10 minutes and 12 hours, more specifically between around 20 minutes and 3 hours, or even more specifically between around 30 and 120 minutes. Thus, an acid may be used to hydrolyse the thin stillage over any of these time periods.

Enzyme hydrolysis may be performed instead of, or together with, chemical hydrolysis. If both approaches are combined they may be performed simultaneously, sequentially or separately. Temperature, time and concentration conditions may need to be adjusted accordingly depending upon the approach taken, as would be appreciated by a skilled person. For example, enzymes may not perform efficiently at low temperatures and may be (irreversibly) denatured at higher temperatures. The type of enzyme employed will depend upon the nature of the thin stillage, which in turn is determined by the fermentation feedstock employed in the bioethanol production process. Typically, glycosidase enzymes are employed in the hydrolyser. In specific embodiments, the hydrolyser contains (in use) a hemi-cellulase and/or a cellulase. Specific examples of such enzymes include glycan hydrolase, E.C.3.2.1 and/or cellulases such as endo beta-glucanases and beta-glucosidase.

The (parent or existing) bioethanol production process can be based upon any suitable fermentation feedstock, which results in thin stillage which acts as the input applied to the apparatus of the invention. The fermentation feedstock may comprise a hemi-cellulose containing material, in particular plant material. Cereal feedstocks may be utilised in the apparatus of the invention. Suitable examples include corn, wheat, barley and sugar beet pulp. The apparatus may thus incorporate, in the fermenter, thermophilic microorganisms

- 17 -

capable of fermenting such hemi-cellulosic sugars derived from plant materials. The feedstock may additionally or alternatively comprise cellulose containing material. Fermentation may thus be of pentose and/or hexose sugars.

- 5 In agreement with the source fermentation feedstock, the non-starch polysaccharides may comprise, consist essentially of or consist of hemicellulose. The non starch polysaccharides may comprise at least around 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% hemicellulose. Similarly, the non starch polysaccharides may comprise at least around 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% cellulose. Cellulose and hemicellulose may
10 make up the total of the non starch polysaccharides. Other polysaccharides may be present depending upon the source of the feedstock, such as pectins, glucans, fructans, glycogen, gums and inulin and may be a source of fermentable sugars such as galactose, arabinase, xylose, glucose (D) and fructose.
- 15 As described in further detail herein, the apparatus of the invention may be specifically adapted to permit fermentation of pentose sugars. Thus, in certain embodiments, partial hydrolysis produces soluble oligomers and monomers which comprise, consist essentially of or consist of pentose sugars. The preferred microorganisms which may be included in the apparatus of the invention can ferment both pentose and hexose sugars and soluble
20 oligomers and monomers may comprise both hexose and pentose sugars. In specific embodiments, the pentose sugars comprise, consist essentially of or consist of xylose and/or arabinose. Soluble oligomers may include disaccharides such as cellobiose and trehalose

- 25 As described above, following partial hydrolysis of the thin stillage (in the hydrolyser), the soluble oligomers and monomers in the partially hydrolysed thin stillage are passed to the fermenter to produce ethanol. Suitable fermentation procedures are well known in the art and readily applied to optimise ethanol production. The fermenter may thus be adapted to enable anaerobic fermentation but may also be adapted to enable fermentation under partially aerobic conditions in certain embodiments, as discussed herein. As discussed
30 above, the fermentation may include fermentation of pentose sugars. Thus, in specific embodiments, the fermenter contains (in use) a microorganism capable of fermenting pentose sugars, which may be a bacterium or yeast, for example. In more specific embodiments, the fermenter contains (in use) a thermophilic microorganism, in particular a thermophilic bacterium capable of fermenting pentose sugars. The thermophilic bacterium
35 may lack lactate dehydrogenase activity. Lactate deficient mutants have previously been shown to be capable of producing increased ethanol yields. Suitable techniques for

inactivating the *ldh* gene (encoding lactate dehydrogenase) are described for example in WO 2007/110608, WO 02/29030 and WO 2006/117536, the relevant disclosure of each of which is incorporated herein in its entirety. Thus, the *ldh* gene may be inactivated through an insertion, deletion or substitution mutation. Lactate production stops and the excess
5 pyruvate diverts mainly into the growth-linked pyruvate formate lyase (PFL) pathway. Thus, the thermophilic bacteria typically express pyruvate formate lyase.

In order to address possible issues of redox imbalance (discussed above), especially under conditions of high sugar levels (produced by the partial hydrolysis), in certain embodiments,
10 the thermophilic bacterium expresses a heterologous NAD-linked (or NAD-dependent) formate dehydrogenase (FDH). Many genes encoding NAD-linked FDH are known in the art (see for example Nanba et al (Biosci. Biotechnol. Biochem. 67(10), 2145-2153 (2003)) and may be employed to transform a suitable thermophilic bacterium. Thus, the thermophilic bacterium may be transformed with an *fdh* gene, in particular an *fdh1* gene. The thermophilic
15 bacterium may incorporate a gene encoding a thermostable NAD-linked formate dehydrogenase in certain embodiments. In other embodiments, the thermophilic bacterium may be transformed with a gene whose nucleotide sequence has been codon optimised to facilitate expression by the thermophilic bacterium. Production of such a thermostable NAD-linked formate dehydrogenase is described in detail in WO 2007/110608, the relevant
20 disclosure of which is incorporated herein in its entirety. In a specific embodiment, the gene encoding an NAD-linked formate dehydrogenase comprises, consists essentially of or consists of the nucleotide sequence set forth as SED ID NO: 1. In a further embodiment, the thermophilic bacterium incorporates a codon optimised (for expression in (Geo) Bacillus) gene encoding a thermostable NAD-linked formate dehydrogenase comprising, consisting
25 essentially of or consisting of the nucleotide sequence set forth as SEQ ID NO: 2. This sequence includes, in addition to the basic thermostable NAD-linked dehydrogenase sequence, promoter and terminator regions and also suitable restriction sites, such as, *Xba*1 sites to facilitate cloning of the gene into a suitable DNA construct.

30 In a still further embodiment the gene encoding an NAD-linked formate dehydrogenase is the *fdh1* gene. The *fdh1* gene may be derived from any suitable source and is preferably codon optimised for expression in the relevant thermophilic bacterium.

The fermentation thus may utilise a synthetic NAD-linked formate dehydrogenase, designed
35 for optimum gene expression due to the use of the codon preferences of the appropriate thermophilic bacterium. The synthetic gene may contain engineered restriction sites to

- 19 -

facilitate insertion into the lactate dehydrogenase gene (in frame relative to the *ldh* promoter). Thereby inactivation of the *ldh* gene and expression of the *fdh* gene are achieved in a single operation. In specific embodiments, the thermostable NAD-linked formate dehydrogenase remains functional at or above a temperature of 60°C. The thermostable enzyme may be encoded by a nucleotide sequence which has been codon optimised for expression in a thermophilic bacterium. The formate dehydrogenase may comprise, consist essentially of or consist of the amino acid sequence set forth as SEQ ID NO: 3, as described in WO 2007/110608, the relevant disclosure of which is incorporated herein in its entirety. Here, a specific thermostable NAD-linked formate dehydrogenase was designed based upon the amino acid sequence of the *Pseudomonas* sp 101 formate dehydrogenase (SEQ ID NO: 3) and through use of optimised codons for *Geobacillus thermoglucosidasius*. The skilled person will appreciate that derivatives of this basic sequence will retain functionality. For example, conservative and semi-conservative substitutions may result in thermostable NAD-linked formate dehydrogenases and these derivatives are intended to fall within the scope of the invention provided they retain effective catalytic activity and thermostability such that they are useful in ethanol production using thermophilic bacteria. Similarly, minor deletions and/or additions of amino acids may produce derivatives retaining appropriate functionality.

In certain embodiments, in order to address the possible issue of redox imbalance, the fermenter is adapted to provide partially aerobic fermentation conditions. As the PDH pathway also operates under aerobic conditions where its operation leads to mainly cell mass production, the metabolic stress mentioned above can be relieved by partial sparging of air, generally performed at an optimum air sparging rate. By optimum air sparging rate is meant a sparging rate that is (just) sufficient to relieve the metabolic stress by allowing a low level of flux through the aerobic PDH pathway. This low level of flux does not, however, allow any significant decrease in the anaerobiosis and hence in the anaerobic PDH flux of the process. This means that there should be no significant decrease in ethanol production levels. Furthermore, because of the severe sensitivity of the PFL pathway towards air, this air sparging may have the additional benefit of reducing the flux through the PFL pathway and further increasing the flux through the anaerobic PDH pathway, but without putting the microorganism under metabolic stress. Thus, in certain embodiments, the fermenter incorporates or is connected to an air sparger to permit sparging of the fermentation. Suitable air sparging rates can be set, for example using an appropriate setting of air flow rate, which rates result in optimal ethanol production levels and/or which minimise or reduce production of formate and acetate. Air sparging may be periodic or continuous and the rate

- 20 -

can be adjusted accordingly. The sparger may sparge the fermentation with air or an alternative oxygen source if desired

Any suitable thermophilic bacterium may be employed in the fermenter included in the apparatus of the invention. In specific embodiments, the thermophilic bacterium is in the family Bacillaceae; more particularly the thermophilic bacterium may be of the genus *Geobacillus*. In specific embodiments, the *Geobacillus* comprises *Geobacillus thermoglucosidasius* or *Geobacillus stearothermophilus*, in particular a strain of *Geobacillus thermoglucosidasius* or *Geobacillus stearothermophilus* transformed with a gene encoding an NAD-linked formate dehydrogenase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic flow diagram of a bioethanol production process and shows the additional elements (dashed boxes) included according to the methods and apparatus of the invention.

Figure 2 shows a similar system to that shown in Figure 1, in which the thin stillage (11) is subjected to a centrifugation step (21) prior to the hydrolysis step (18) in order to remove suspended solids, predominantly protein, from the thin stillage (11). Again aspects of the invention are shown in dashed boxes relative to the components of the parent or existing bioethanol production process.

Figure 3 shows an alternative embodiment to Figure 2 in which the solids may be passed to a separate dryer (31) and then sold as a separate, high protein content, product (32).

Figure 4 is a chromatogram of untreated thin stillage.

Figure 5 is a chromatogram of thin stillage hydrolysed with nitric acid.

Figure 6 is an overlay of the chromatograms of untreated (dashed line) and nitric acid treated (line) thin stillage samples.

Figure 7 is a chromatogram of thin stillage treated with enzymes at pH 5.0 and a temperature of 50°C for 24 hours.

Figure 8 is an overlay of the chromatograms of untreated (dashed line) and enzyme treated (line) thin stillage samples.

5 DESCRIPTION OF THE PREFERRED EMBODIMENTS

With reference to Figure 1, a bioethanol production process is shown incorporating the additional process elements of the invention. The feedstock passes (at the process stream inlet) to and through hammer mills to produce coarse particles known as “meal”. This is the milling step (1). In the mashing step (2), water and enzymes (such as amylase) are added to the meal in a mixing tank to produce what is known as “mash”. As part of the liquefaction step (3), the mash is then cooked in a cooking system to around 100°C. This may involve use of jet-cookers that inject steam into the mash. This liquefies the starch and reduces the levels of bacteria in the mash. Enzymes are then added to the liquefied mash to convert starches in the mash to simple sugars such as dextrose. In the mash cooling step (4), the mash is passed through a series of heat exchangers to cool the mash, to a temperature of around 30°C if yeast fermentation is to be performed or higher, such as around 50°C, if thermophilic bacteria (as described herein) are to be used, prior to onward transmission into the fermenter. As the fermenter fills additional enzymes (e.g. glucoamylase) may be added to complete the breakdown of starch into simple sugars (e.g. glucose). This is referred to as saccharification. In the fermentation step (5) a suitable microorganism, such as a yeast or bacterium is added to the mash to convert sugars to ethanol (by saccharification fermentation). The process produces a “beer solution” that contains alcohol and non-fermentable solids, together with heat and carbon dioxide. The other components of the feedstock (protein, oil, etc.) remain largely unchanged during the fermentation process. In most dry-grind ethanol plants, the fermentation process occurs in batches. A fermentation tank is filled, and the batch ferments completely before the tank is drained and refilled with a new batch. The up-stream processes (milling, mashing, liquefaction, and saccharification) and downstream processes (distillation and recovery) occur continuously (grain is continuously processed through the equipment). Thus, dry-grind facilities of this design usually have at least three fermenters (tanks for fermentation) where, at any given time, one is filling, one is fermenting (usually for at least 48 hours), and one is emptying and resetting for the next batch. Carbon dioxide is also produced during fermentation. If the carbon dioxide is not recovered it is simply released from the fermenters to the atmosphere. Preferably, the carbon dioxide is recovered. If recovered, this carbon dioxide can be compressed and sold for carbonation of soft drinks or frozen into dry ice for cold product

- 22 -

storage and transportation. After the fermentation is complete, the fermented corn mash (now called "beer") is emptied from the fermenter into a beer well (6). The beer well (6) stores the fermented beer between batches and supplies a continuous stream of material to the ethanol recovery steps, including distillation (7).

5

During distillation (7) the beer solution is continuously pumped through a multi-column system that separates the alcohol (8) and the whole stillage (9). Conventional distillation/rectification systems can produce ethanol at 92-95% purity. The residual water is then removed using molecular sieves that selectively adsorb the water from an ethanol/water vapour mixture, resulting in nearly pure ethanol (>99% or 200 proof). The resultant ethanol can be stored, for onward shipment to fuel terminals (not shown) and can be denatured using a small percentage of automotive fuel (not shown).

10

The residual water and solids that remain after the distillation process are called whole stillage (9). This whole stillage (9) is then centrifuged to separate (10) the liquid or solubles (thin stillage (11)) from the solid fragments of the kernel or coarse grain solids (wet cake or distillers' grains (14)). The thin stillage (11) passes through evaporators (12) to remove a significant portion of the water to produce thickened syrup (13). Often, the syrup (13) is blended with the wet cake (14) or distillers' grains and dried in a dryer (15) to produce an animal feed called "distillers' dried grains with solubles" (DDGS) (16). In some embodiments, such as when markets for the feed product are close to the plant, the by-product may be sold without drying (15) as distillers' grains or wet distillers' grains (not shown).

15

20

In conventional bioethanol processes, some of the thin stillage (11) may be recycled to the beginning of the dry-grind process. This is termed backset (17) and may or may not be incorporated into the processes of the invention. In the present invention, a proportion of the thin stillage (11) is subjected to a hydrolysis step (18). Hydrolysis may be chemical and/or enzymatic and permits conversion of non starch polysaccharides to soluble oligomers and monomers. The hydrolysed thin stillage is then subjected to a new fermentation (19) of the soluble oligomers and monomers produced by the hydrolysis (18). This new fermentation (19) produces additional ethanol. The "beer solution" produced by the new fermentation is recycled back to the front end of the bioethanol plant, at any stage prior to the distillation step (7). This permits the additional ethanol to be recovered by distillation (7) using the existing equipment in the bioethanol plant. This new process has a number of benefits. More ethanol is produced as a proportion of the feedstock. The additional hydrolysis and fermentation can improve the composition of the by-products of the process by removing anti

25

30

35

nutritives. By utilising the thin stillage more effectively, drying capacity is freed up in the overall system (which is often the limiting factor in overall production efficiency).

Figure 2 shows a similar system to that shown in Figure 1. No separate backset is employed in this embodiment. In this embodiment, the thin stillage (11) is subjected to a centrifugation step (21) prior to the hydrolysis step. The centrifugation step (21) is utilised in order to remove suspended solids, predominantly protein, from the thin stillage (11). The centrifugation step (21) may utilise disk stack centrifugation as it permits separation of smaller particles based upon lower solids concentrations than decanter centrifugation. The liquid portion passes to the hydrolysis step (18), whereas the deposited solids (22) are passed to the dryer (15). The additional protein (22) recovered from the centrifugation step (21) may improve the value of the DDGS (16).

Figure 3 shows an alternative embodiment to Figure 2 in which the solids may be passed to a separate dryer (31) and then sold as a separate, high protein content, product (32).

EXPERIMENTAL EXAMPLES

The invention will be further described with reference to the following non-limiting experimental examples:

EXAMPLE 1 - Acid and Enzyme hydrolysis of Thin Stillage

Introduction

Thin stillage was obtained from a bioethanol process (Ensus). Figures 1 and 2 show a bioethanol production process and the point where thin stillage is produced in the process. Thin stillage is the semi-solid residue stream of the ethanol process and it is obtained after removing wet cake from the residue. The thin stillage is expected to be starch and glucose free and the carbohydrates will mainly be cellulosic and hemicellulosic residues. It is also expected that its hydrolysis, under optimum conditions, will release most of the sugars from these materials.

Materials and Methods

Thin stillage

Thin stillage was made available by Ensus from their bioethanol plant (See the introduction section and Figures 1 and 2).

Acid hydrolysis of thin stillage

- 5 In a 100 ml Duran bottle containing about 12.5 ml of thin stillage, 0.125 ml of concentrated nitric or 0.136 ml of sulphuric acid was added and hydrolysed (autoclaved) at 121 °C for 30 minutes.

Enzyme hydrolysis of thin stillage

- 10 In 250 ml conical flasks containing about 50 – 100 ml of thin stillage (adjusted to pH 4 or 5); different levels of enzyme(s) according to Table 1 below were added. The flasks were then incubated at 60 °C for various intervals. Samples were drawn from the flasks and analysed by HPLC.

- 15 Table 1 – Enzymes used in hydrolysis of thin stillage. Enzyme concentration was calculated on the basis of the solid contents of thin stillage (TS) as 8% w/v.

Genencor Enzymes	Recommended concentrations (g/g dry weight)	Amount added/100 ml TS*
Accellerase Duet	0.05-0.25	2 ml
Accellerase 1500	0.05-0.25	2 ml
Optimash BG	0.025-0.05	0.72ml
Optimash TBG	0.025-0.05	0.72ml
Accellerase XY	0.005-0.05	0.4 ml
Accellerase XC	0.0125-0.125	1.2 ml

Dried insoluble solids in thin stillage

- 20 Total solids in the thin stillage were calculated by centrifuging the thin stillage at 4000 rpm for 10 to 20 minutes, removing the supernatant and drying it at 65°C for 48 hours.

Dried soluble + insoluble solids in thin stillage

Thin stillage was kept at 120 °C until a constant weight was obtained (in about 8 minutes).

- 25 Analysis of thin stillage

Hydrolysed and unhydrolysed thin stillage was doubly centrifuged at 14000 rpm for 5 minutes and then filtered through 0.2 micron filter and analysed through a Dionex HPLC machine fitted with Dionex CarboPAC PA1 column kept at ambient temperature and eluted with

- 25 -

gradient mobile phase [100% A (50mM NaOH) for 20 minutes followed by 100% B (250mM NaOAc/250mM NaOH) for 10 minutes followed by 15 minutes column regeneration with 100% A. Total time is 45 minutes at the flow rate of 1 ml/min.

- 5 Alternatively, the samples were analysed using Shimadzu HPLC machine fitted with Bio-Rad Aminex-HPX-87H column kept at 65°C temperature and eluted with 5 mM sulphuric acid for 25 minutes at the flow rate of 0.6 ml/min.

Results

10

Total solids in thin stillage

Total solids (from soluble + insoluble) in the thin stillage were found to be between 60 and 85 g/l when the thin stillage was dried at 120°C for 8 minutes and most of the thin stillage batches had around 80 g/l total solid contents. Total insoluble solids were found to be about 15 45 g/l when the solids were first separated from the residues and then the residue was dried at 65°C for 48 hours.

Carbohydrate Release by Acid Hydrolysis of thin stillage

The results in Table 2 and Figure 4 show that very small amount of sugars were present in 20 the thin stillage (only 1.7 g/l of monomers sugars and 3 g/l of dimer sugars). Simple autoclaving released only a very small amount of monomer sugars (about 1.8 g/l). However, acid hydrolysis released a significant amount of sugars from the thin stillage. Hydrolysis of the stillage at 121°C for 30 minutes with 1% nitric acid increased the soluble monomer 25 g/l (Figures 5 and 6). A similar reduction in the dimer levels was achieved with 1% sulphuric acid treatment while the increase in the monomer sugars to 22.5 g/l was marginally less than that achieved with nitric acid.

Carbohydrate Release by Enzyme Hydrolysis of thin stillage

30 Qualitative results presented in Figures 7 and 8 clearly indicate that a significant amount of monomer sugars were released during the enzymatic hydrolysis. The quantitative results of the hydrolysis of thin stillage with different enzymes are shown in Table 3. Accellerase 1500, Accellerase Duet, Optimash GB and Optimash TBG release significant amounts of sugars and up to about 25 g/l of sugar could be released from the thin stillage. While Accellerase XC 35 and Accellerase XY also release some sugars from the thin stillage.

Conclusions

- The dried insoluble solid content of the thin stillage is about 45 g/l
- The dried solid content (soluble + insoluble) of the thin stillage is between 60 and 85 g/l
- About 21 g/l sugars with 1% sulphuric and about 23 g/l sugars with 1% nitric acid were released from the hydrolysis of the thin stillage.
- Enzymatic hydrolysis also released a significant amount of sugars from the thin stillage.

Table 2: Carbohydrate levels (g/l) in thin stillage with and without treatment

Treatment	Glucose	Xylose	Arabinose	Others	Total Mono.
None	0	0.73	1.01	3.01	1.74
121 °C - 30 min	0.29	2.14	1.07	3.2	3.50
121 °C - 30 min with 1% HNO ³	6.76	13.36	5.84	0.08	25.96
121 °C - 30 min with 1% H ₂ SO ₄	5.54	11.95	5.01	0.07	22.50

Mono. = monomer sugars

The solids in thin stillage were 45 g/l.

Table 3: Carbohydrate levels (g/l) in thin stillage released with different enzymes (Genencor) at 60°C and pH 5 after 24 hours.

Enzymes	Amount Added/ 100 mL TS	Arabinose (g/L)	Glucose (g/L)	Xylose (g/L)	Total sugars (g/L)
Accellerase Duet	0.125mL	0.69	12.11	4.39	23.19
Accellerase 1500	2mL	5.5	17.9	2	25.5
Optimash BG*	0.72mL	1.76	16.10	2.25	20.11
Optimash TBG*	0.72mL	2.45	19.26	3.42	25.12
Accellerase XY	0.4mL	6	10.9	0	17.3
Accellerase XC**	1.2mL	3.7	3.64	0.48	11.8

*, Hydrolysis was performed at pH 4 (instead of pH 5) and at 50°C (instead of 60°C)

**, Hydrolysis was carried out for 6 hours (instead of 24 hours).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. Moreover, all embodiments described herein are considered to be broadly applicable and combinable with any and all other consistent embodiments, as appropriate.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

5 SEQUENCE LISTING

SEQ ID NO: 1

	atggcaaaag tactttgCGT tctttatgat gatccggtag atggctatcc gaagacgtat	60
	gcccGagatg atttaccgaa gatagatcac tatccaggag ggcaaacatt gccgacgccg	120
10	aaagcaattg acttcacgcc tgggcaattg ttaggaagcg tatctggcga gctgggactt	180
	agaaagtatc ttgagtccaa tggacatacg ttagtggtaa ctagcgataa ggatgggccca	240
	gactcagtgt ttgaacggga gttagtggat gccgatgttg tcattagtca accgttcttg	300
	cctgcataatc ttacgccgga aagaatcgca aaagcgaaga acttgaaact agccctgaca	360
	gcaggaattg gaagcgatca tgtggatttg caaagcgcta ttgatcgcaa tgttaccgtg	420
15	gcagaggtga catattgtaa ttctattagt gtagctgagc atgtggtaat gatgatttta	480
	tccttagtta gaaattactt gccgagccac gaatgggctc gtaaaggcgg gtggaatatt	540
	gcagattgcg tttctcatgc ttatgattta gaggcgatgc atgttggcac ggttgcggcg	600
	ggacgtatag gcttggcagt cttgcgtcga ctagcaccgt ttgacgttca ttacactat	660
	actgatcgac atcgtcttcc agagtccgtt gagaaagaac ttaacttaac ctggcatgca	720
20	acgcgtgaag atatgtatcc ggtgtgtgac gtggtaacat tgaattgccg gttacatcct	780
	gaaactgaac acatgatcaa tgacgaaacg ttgaaactgt ttaaacgagg cgcttatatc	840
	gtaaacacag ccagagggaa actttgtgat cgggatgctg tagccagagc acttgagagc	900
	ggacgcttag ccgggtatgc aggcgacgtg tggtttccac aacctgcccc gaaagatcat	960
	ccgtggagaa cgatgccgta taatggaatg acgccacata tttcaggcac tacgttaaca	1020
25	gcacaagcac gttatgcggc cggcaccCGT gaaattcttg agtgcttctt cgaaggccgt	1080
	ccgatccgag atgaatatTT gattgtacaa ggtggcgcat tagctgggac aggagcacat	1140
	agttatagca aaggcaatgc tacgggagggc	1170

SEQ ID NO: 2

30	gcctctagaa gggcaatctg aaaggaaggg aaaattcctt tcggattgtc cttttagtta	60
	tttttatggg gagtgaatat tatataggca ttacggaaat gataatggca gagttttttc	120
	atttattaga ctgcttgatg taattggatg tgatgataca aaaataatgt tgtgtaaaca	180
	aaatgttaac aaaaaagaca aatttcattc atagttgata cttgataaag attgtgaaat	240
	aatgcacaat atatcaatgt atgagcagtt tcacaaattc attttttTGA aaggatgaca	300
35	gacagcgatg gcaaaagtac tttgcgttct ttatgatgat ccggtagatg gctatccgaa	360
	gacgtatgcc cgagatgatt taccgaagat agatcactat ccaggagggc aaacattgcc	420
	gacgccgaaa gcaattgact tcacgcctgg gcaattgtta ggaagcgtat ctggcgagct	480
	gggacttaga aagtatcttg agtccaatgg acatacgtta gtggtaacta gcgataagga	540

- 28 -

	tggggccagac	tcagtgtttg	aacggggagtt	agtggatgcc	gatgttgtca	ttagtcaacc	600									
	gttctggcct	gcatacttta	cgccggaaaag	aatcgcaaaa	gcgaagaact	tgaaactagc	660									
	cctgacagca	ggaattggaa	gcgatcatgt	ggatttgcaa	agcgctattg	atcgcaatgt	720									
5	taccgtggca	gaggtgacat	attgtaattc	tattagtgtg	gctgagcatg	tggtaatgat	780									
	gattttatcc	ttagttagaa	attacttgcc	gagccacgaa	tgggctcgta	aaggcgggtg	840									
	gaatattgca	gattgcgttt	ctcatgctta	tgatttagag	gcgatgcatg	ttggcacggt	900									
	tgcggcggga	cgtataggct	tggcagtctt	gcgtcgacta	gcaccgtttg	acgttcattt	960									
	acactatact	gatcgacatc	gtcttccaga	gtccgttgag	aaagaactta	acttaacctg	1020									
10	gcatgcaacg	cgtgaagata	tgtatccggt	gtgtgacgtg	gtaacattga	attgcccgtt	1080									
	acatcctgaa	actgaacaca	tgatcaatga	cgaaacgttg	aaactgttta	aacgaggcgc	1140									
	ttatatcgta	aacacagcca	gagggaaaact	ttgtgatcgg	gatgctgtag	ccagagcact	1200									
	tgagagcgga	cgcttagccg	ggtatgcagg	cgacgtgtgg	tttccacaac	ctgccccgaa	1260									
	agatcatccg	tggagaacga	tgccgtataa	tggaatgacg	ccacatattt	caggcactac	1320									
15	gttaacagca	caagcacggt	atgcggccgg	caccctgtaa	attcttgagt	gcttcttcga	1380									
	aggccgtccg	atccgagatg	aatatttgat	tgtacaaggt	ggcgcattag	ctgggacagg	1440									
	agcacatagt	tatagcaaag	gcaatgctac	gggaggcagc	gaggaagcag	ctaaatttaa	1500									
	gaaagcgggt	taacacagca	ggggctgac	ggcccctggt	atgtttcatt	ctagagcc	1558									
20	SEQ ID NO: 3															
	Met	Ala	Lys	Val	Leu	Cys	Val	Leu	Tyr	Asp	Asp	Pro	Val	Asp	Gly	Tyr
	1				5					10					15	
	Pro	Lys	Thr	Tyr	Ala	Arg	Asp	Asp	Leu	Pro	Lys	Ile	Asp	His	Tyr	Pro
					20					25					30	
25	Gly	Gly	Gln	Thr	Leu	Pro	Thr	Pro	Lys	Ala	Ile	Asp	Phe	Thr	Pro	Gly
			35							40					45	
	Gln	Leu	Leu	Gly	Ser	Val	Ser	Gly	Glu	Leu	Gly	Leu	Arg	Lys	Tyr	Leu
			50							55					60	
	Glu	Ser	Asn	Gly	His	Thr	Leu	Val	Val	Thr	Ser	Asp	Lys	Asp	Gly	Pro
30	65					70					75					80
	Asp	Ser	Val	Phe	Glu	Arg	Glu	Leu	Val	Asp	Ala	Asp	Val	Val	Ile	Ser
						85					90					95
	Gln	Pro	Phe	Trp	Pro	Ala	Tyr	Leu	Thr	Pro	Glu	Arg	Ile	Ala	Lys	Ala
						100					105				110	
35	Lys	Asn	Leu	Lys	Leu	Ala	Leu	Thr	Ala	Gly	Ile	Gly	Ser	Asp	His	Val
						115					120				125	
	Asp	Leu	Gln	Ser	Ala	Ile	Asp	Arg	Asn	Val	Thr	Val	Ala	Glu	Val	Thr
						130					135				140	
	Tyr	Cys	Asn	Ser	Ile	Ser	Val	Ala	Glu	His	Val	Val	Met	Met	Ile	Leu

- 29 -

[illegible]

35

Claims

1. A method for processing the thin stillage stream resulting from a bioethanol
5 production process comprising:
 (a) partial hydrolysis of the thin stillage stream resulting from the bioethanol
production process, which partial hydrolysis converts non starch polysaccharides to soluble
oligomers and monomers,
 (b) fermentation of the soluble oligomers and monomers in the partially
10 hydrolysed thin stillage to produce ethanol
 (c) recycling of the product stream resulting from steps (a) and (b) to the front
end of the bioethanol production process such that the ethanol produced in step (b) is
recovered as part of a bioethanol recovery process in the bioethanol production process.
- 15 2. The method of claim 1 wherein between 10 and 60% of the thin stillage stream
resulting from the bioethanol production process is utilised in step (a).
3. The method of claim 1 or claim 2 comprising a centrifugation step applied to the thin
stillage in order to remove suspended solids, in particular protein, from the thin stillage.
- 20 4. The method of claim 3 wherein the centrifugation step is performed using a disk stack
centrifuge.
5. The method of claim 3 or claim 4 wherein the centrifugation step is performed at
25 any one or more of the following stages: prior to step (a), after step (a) but before step (b)
and after step (b) but before step (c).
6. The method of any of claims 3 to 5 wherein the solids, in particular protein
removed by performing the centrifugation step are subject to a drying process, and
30 optionally then sold as a dried product.
7. The method of any preceding claim wherein recycling of the product stream
resulting from steps (a) and (b) to the front end of the bioethanol production process
comprises recycling of the product stream resulting from steps (a) and (b) to any one or more

- 31 -

of the process stream inlet, milling, mashing, liquefaction, mash cooling, fermentation, beerwell and distillation.

8. The method of any preceding claim wherein the bioethanol production process
5 relies upon a fermentation feedstock comprising a hemi-cellulose containing plant material.

9. The method of any preceding claim wherein the partial hydrolysis comprises up to around 75% hydrolysis of the non starch polysaccharides.

10. The method of any preceding claim wherein the non starch polysaccharides comprise
10 at least 50% hemicellulose.

11. The method of any preceding claim wherein the soluble oligomers and monomers
15 comprise pentose sugars.

12. The method of claim 11 wherein the pentose sugars comprise xylose and/or
arabinose.

13. The method of any preceding claim wherein the partial hydrolysis is performed
20 chemically and/or enzymatically.

14. The method of claim 13 wherein the chemical partial hydrolysis employs an acid.

15. The method of claim 14 wherein the acid is sulphuric acid, nitric acid or hydrochloric
25 acid.

16. The method of claim 15 wherein the acid is employed at a concentration of around 0.5-5% acid.

17. The method of claim 15 or 16 wherein the acid is employed at a temperature of
30 between around 100 and 150 degrees Celsius.

18. The method of any of claims 15 to 17 wherein the acid is employed for a period of
35 between around 20 and 120 minutes.

- 32 -

19. The method of claim 13 wherein the enzyme or enzymes comprise a hemi-cellulase and/ or a cellulase.
20. The method of any preceding claim wherein the fermentation of the soluble oligomers and monomers in step (b) is carried out under partially aerobic conditions.
21. The method of claim 20 wherein partially aerobic conditions are achieved by air sparging.
22. The method of any preceding claim wherein the fermentation of the soluble oligomers and monomers in step (b) is carried out by a thermophilic bacterium.
23. The method of claim 22 wherein the thermophilic bacterium lacks lactate dehydrogenase activity.
24. The method of claim 22 or 23 wherein the thermophilic bacterium expresses a heterologous NAD-linked formate dehydrogenase.
25. The method of any of claims 22 to 24 wherein the thermophilic bacterium is of the genus *Geobacillus*.
26. The method of claim 25 wherein the *Geobacillus* comprises *Geobacillus thermoglucosidasius* or *Geobacillus stearothermophilus*.
27. The method of any preceding claim which results in production of a co-product with improved nutritional content, wherein the improved nutritional content comprises one or more of decreased levels of pentose sugars, increased protein concentration, decreased fibre concentration, decreased levels of soluble oligomers and monomers, decreased levels of reducing sugars.
28. An animal feed product produced according to the method of any preceding claim.
29. An apparatus for processing the thin stillage stream resulting from a bioethanol production process comprising:
- (a) a hydrolyser connectable or connected to a thin stillage stream resulting from a bioethanol production process for partial hydrolysis of the thin stillage stream,

- 33 -

which partial hydrolysis converts non starch polysaccharides to soluble oligomers and monomers

5 (b) a fermenter connectable or connected to the hydrolyser for fermentation of the soluble oligomers and monomers in the partially hydrolysed thin stillage to produce ethanol (additional to that produced by the bioethanol production process)

10 (c) a recycler connectable or connected to the fermenter for recycling of the product stream resulting from steps (a) and (b) to the front end of the bioethanol production process such that the additional ethanol produced in step (b) is recovered as part of a bioethanol recovery process in the bioethanol production process.

10

30. The apparatus according to claim 29 further comprising:

(d) a centrifuge for removal of suspended protein from thin stillage and which is connectable or connected to the hydrolyser and/or the fermenter.

15 31. The apparatus according to claim 30 wherein the recycler is connected downstream of the centrifuge, which in turn is connected downstream of the fermenter such that the thin stillage stream passes from the fermenter to the centrifuge and the liquid stream from the centrifuge passes to the recycler following centrifugation.

20 32. The apparatus according to claim 30 wherein the centrifuge is connected upstream of the hydrolyser and thus removes suspended protein from the thin stillage stream prior to partial hydrolysis of the resultant stream.

25 33. The apparatus according to claim 30 wherein the centrifuge is connected downstream of the hydrolyser and thus removes suspended protein from the thin stillage stream following partial hydrolysis thereof.

30 34. The apparatus of claim 33 wherein the centrifuge is connected upstream of the fermenter such that the thin stillage stream passes from the hydrolyser to the centrifuge and the liquid stream from the centrifuge passes to the fermenter following centrifugation.

35 35. The apparatus of any one of claims 29 to 34 wherein the recycler is connectable or connected to the front end of the bioethanol production process at any one or more of the process stream inlet, the miller, the masher, the liquefier, the mash cooler, the fermenter, the beerwell and the distillation inlet.

- 34 -

36. The apparatus of any one of claims 29 to 35 wherein the hydrolyser contains an acid.
37. The apparatus of claim 36 wherein the acid is sulphuric acid, nitric acid or hydrochloric acid.
- 5 38. The apparatus of claim 36 or 37 wherein the acid is at a concentration of around 0.5-5% acid.
39. The apparatus of any one of claims 36 to 38 wherein the hydrolyser contains a
10 control element to achieve a temperature of between around 100 and 150 degrees Celsius or wherein the apparatus contains a separate heating vessel to heat the acid to a temperature of between around 100 and 150 degrees Celsius.
40. The apparatus of any one of claims 36 to 39 further comprising sensors to permit
15 partial hydrolysis to proceed for a period of between around 20 and 120 minutes.
41. The apparatus of any one of claims 29 to 40 wherein the hydrolyser contains one or more enzymes, which optionally comprise a hemi-cellulase and/or a cellulase.
- 20 42. The apparatus of any one of claims 29 to 41 wherein the fermenter is adapted to provide partially aerobic fermentation conditions.
43. The apparatus of claim 42 wherein the fermenter incorporates or is connected to an
25 air sparger to permit sparging of the fermentation.
44. The apparatus of any one of claims 29 to 43 wherein the fermenter contains a thermophilic bacterium.
45. The apparatus of claim 44 wherein the thermophilic bacterium lacks lactate
30 dehydrogenase activity.
46. The apparatus of claim 44 or 45 wherein the thermophilic bacterium expresses a heterologous NAD-linked formate dehydrogenase.
- 35 47. The apparatus of any one of claims 44 to 46 wherein the thermophilic bacterium is of the genus *Geobacillus*.

- 35 -

48. The apparatus of claim 47 wherein the *Geobacillus* comprises *Geobacillus thermoglucosidasius* or *Geobacillus stearothermophilus*.

5 49. The apparatus of any one of claims 30 to 48 wherein the centrifuge is connectable or connected to a dryer and wherein the dryer dries the solid stream obtained from centrifugation.

10 50. A method for processing the thin stillage stream resulting from a bioethanol production process substantially as described herein with reference to the accompanying drawings.

15 51. An animal feed product substantially as described herein with reference to the accompanying drawings.

52. An apparatus for increasing ethanol yields in a bioethanol production process substantially as described herein with reference to the accompanying drawings.

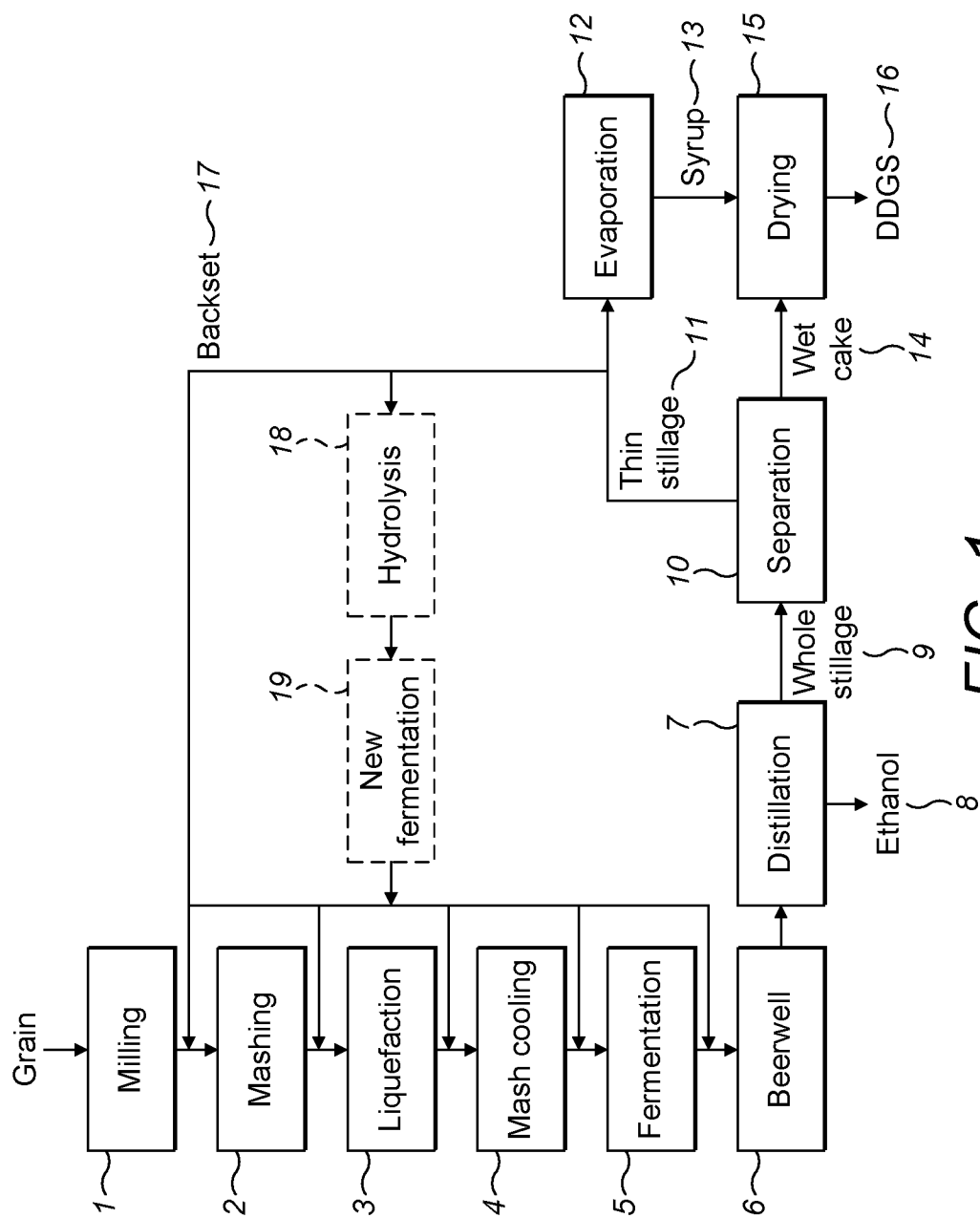


FIG. 1

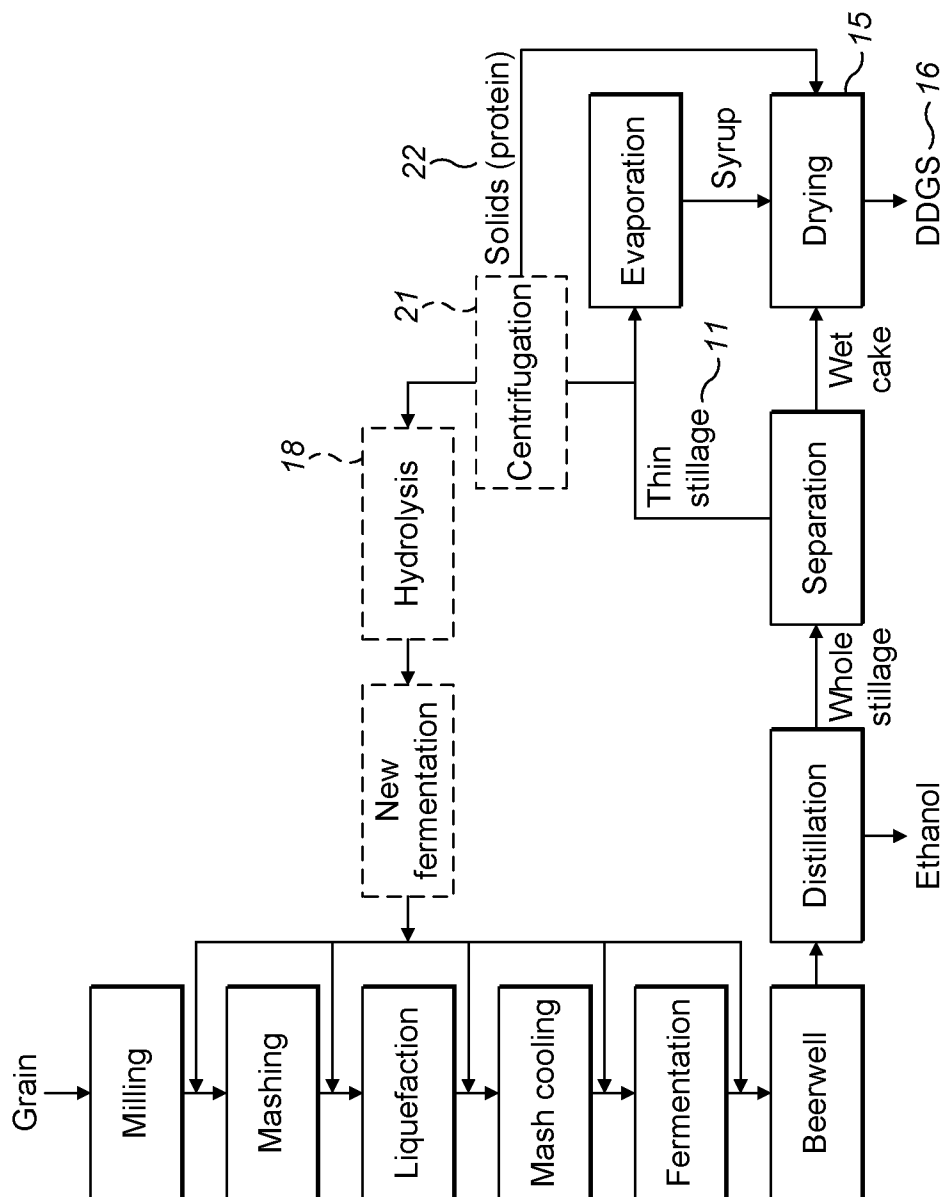


FIG. 2

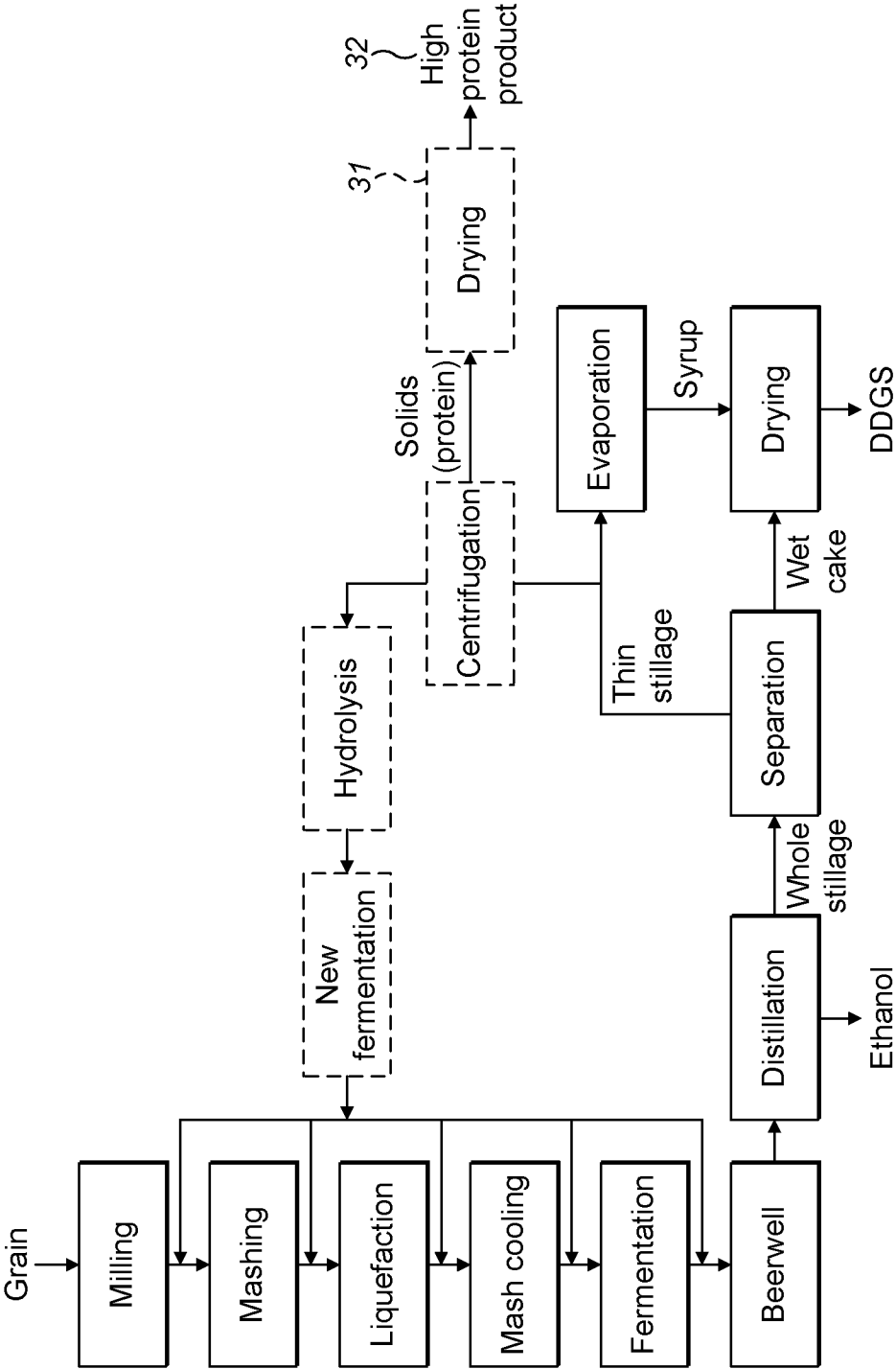


FIG. 3

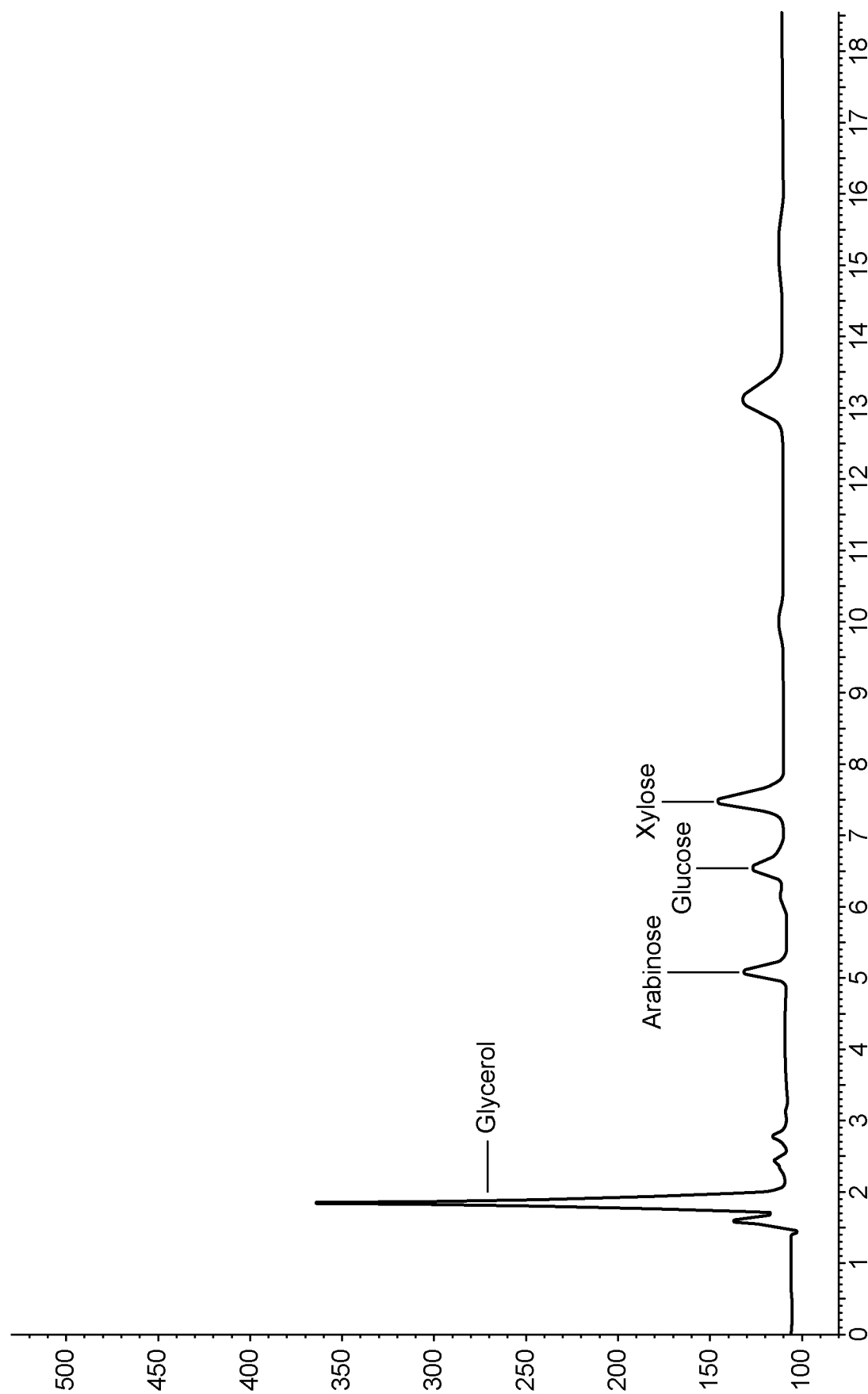


FIG. 4

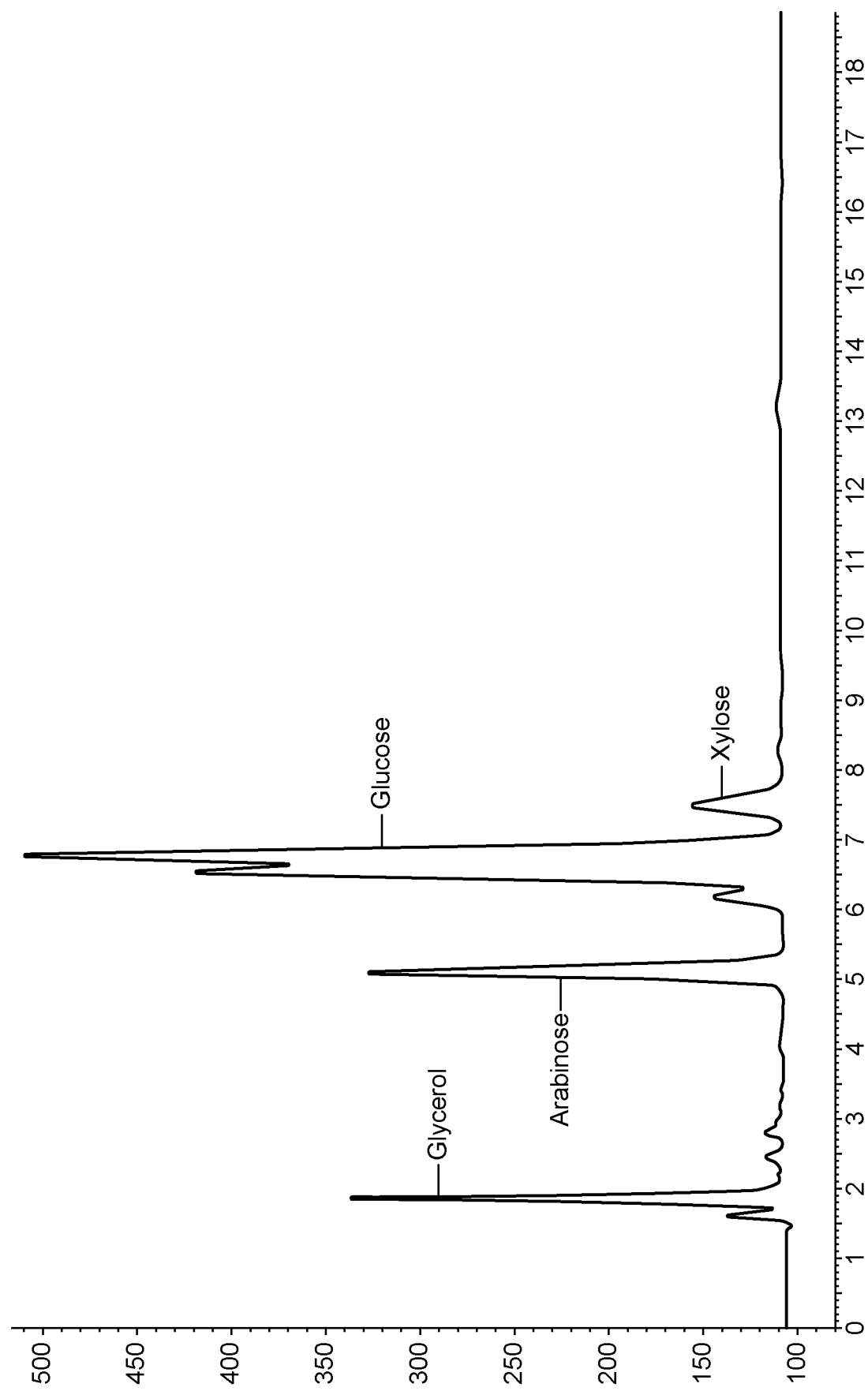


FIG. 5

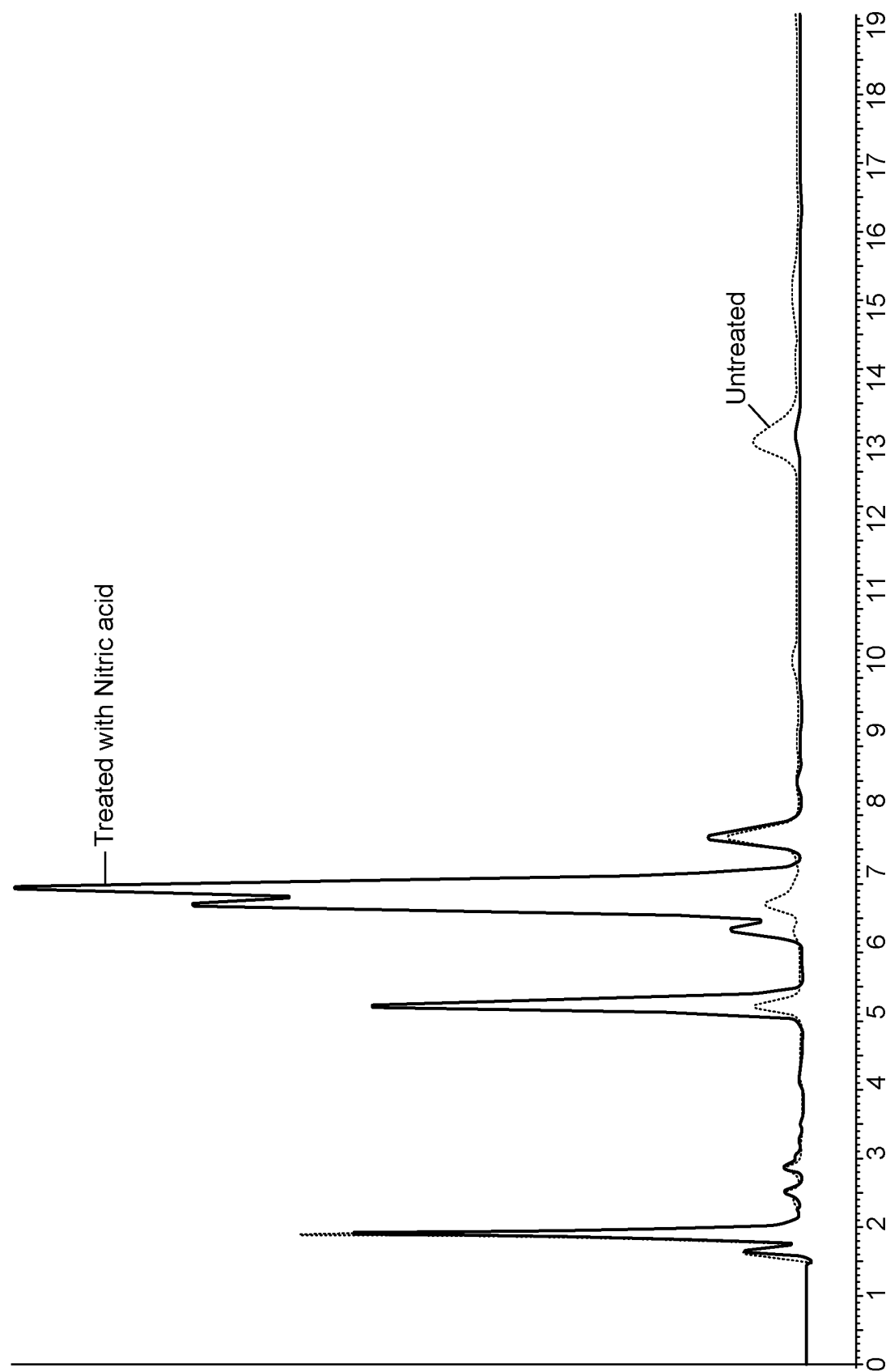


FIG. 6

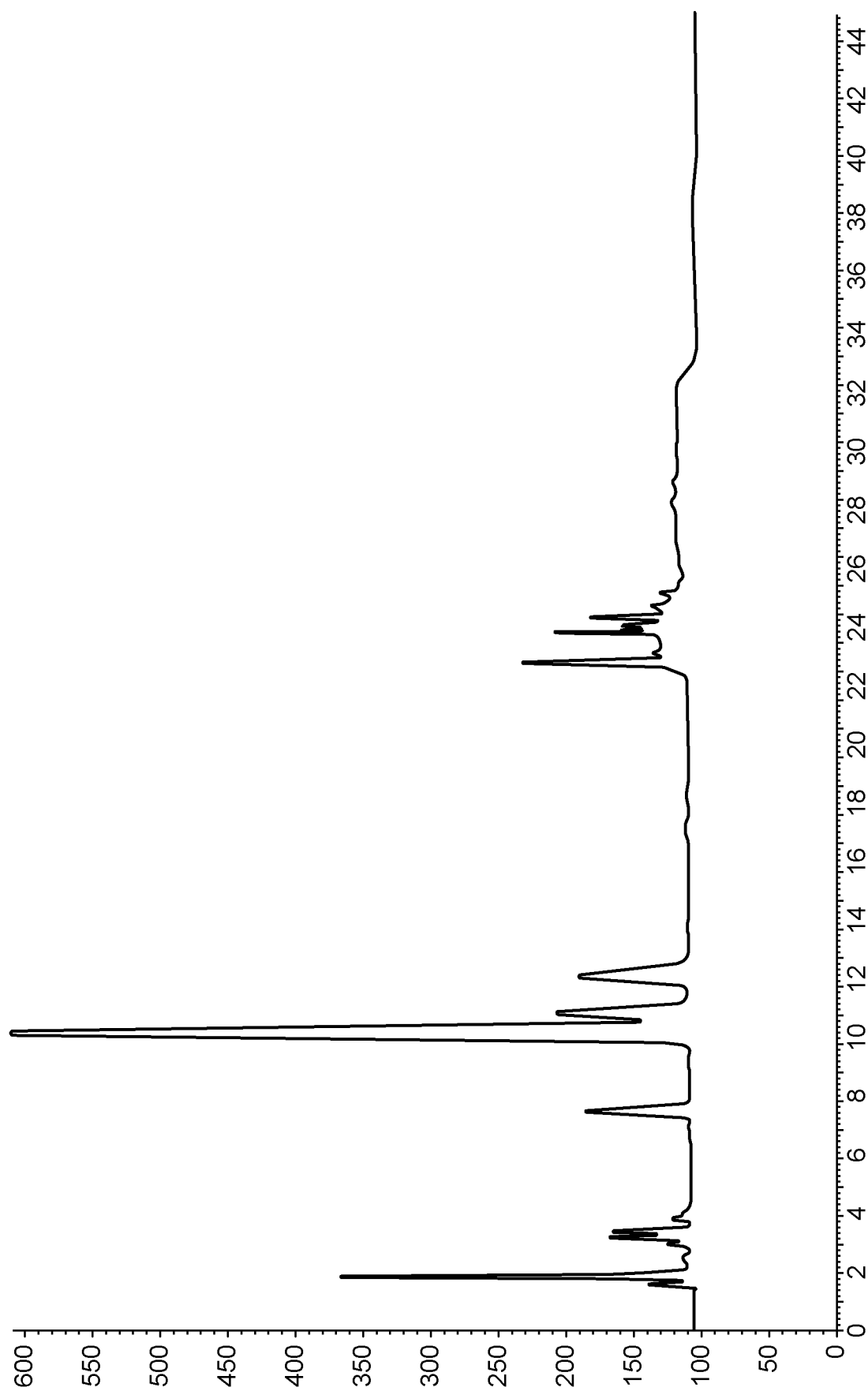


FIG. 7

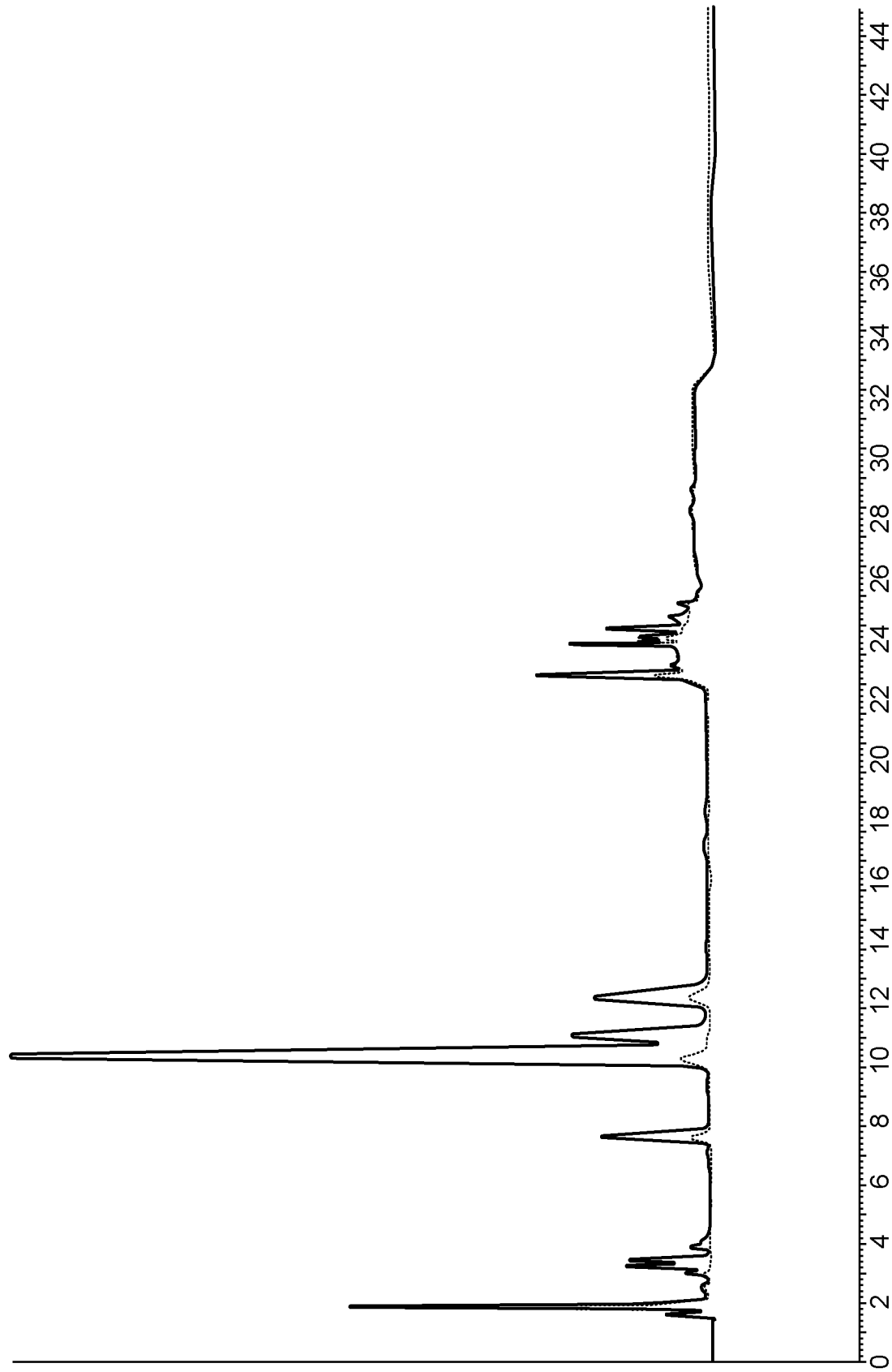


FIG. 8