

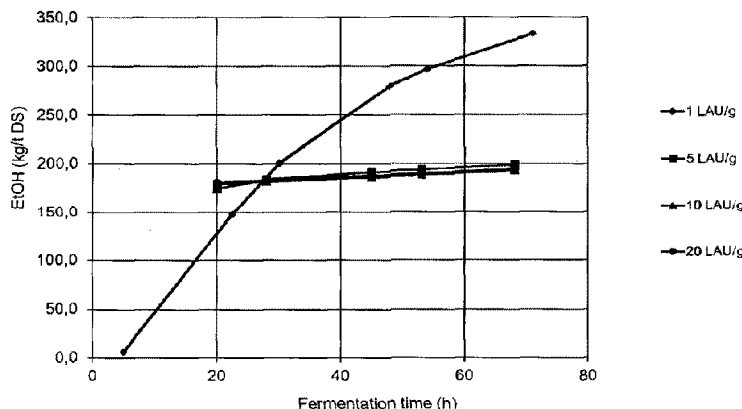


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(54) Titre : PROCEDE DE SACCHARIFICATION ET DE FERMENTATION EN SIMULTANE DE PERMEAT DE LACTOSERUM
(54) Title: PROCESS FOR SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF WHEY PERMEATE

SSF-fermentation of whey permeate(20% DS)



(57) **Abrégé/Abstract:**

A process for producing ethanol from lactose containing substrates, comprising simultaneously saccharifying the substrate to produce monosaccharide and fermenting the monosaccharide to produce ethanol at a pH from 3.5 - 5.5, using a fermenting organism, wherein saccharification is carried out in the presence of a lactase, and wherein the fermenting organism is a *Saccharomyces* sp., and the ratio between the incubation time required for obtaining at least 90% hydrolysis of the lactose present in the substrate (t_1) and the total fermentation time (t_2) is in the range of 0.1 to 1, and the *Saccharomyces* sp. is added in amounts that will result in an ethanol yield of at least 70 % w/w of the theoretical ethanol yield from lactose by the end of fermentation.

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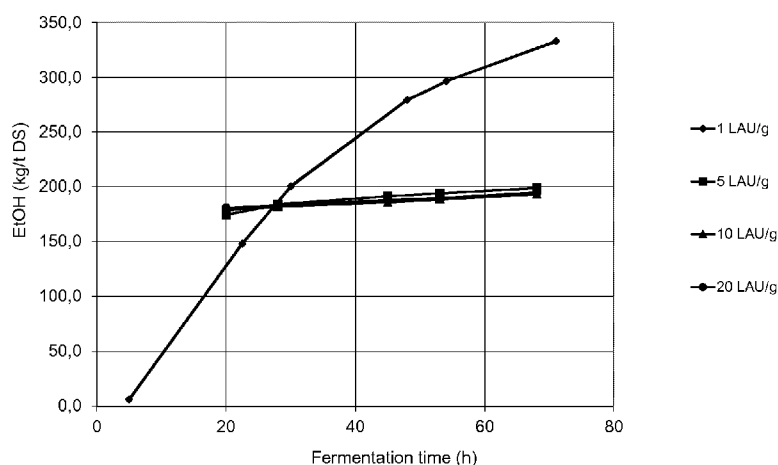
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Figure 4.

SSF-fermentation of whey permeate(20% DS)



(57) Abstract: A process for producing ethanol from lactose containing substrates, comprising simultaneously saccharifying the substrate to produce monosaccharide and fermenting the monosaccharide to produce ethanol at a pH from 3.5 - 5.5, using a fermenting organism, wherein saccharification is carried out in the presence of a lactase, and wherein the fermenting organism is a *Saccharomyces* sp., and the ratio between the incubation time required for obtaining at least 90% hydrolysis of the lactose present in the substrate (t_1) and the total fermentation time (t_2) is in the range of 0.1 to 1, and the *Saccharomyces* sp. is added in amounts that will result in an ethanol yield of at least 70 % w/w of the theoretical ethanol yield from lactose by the end of fermentation.

PROCESS FOR SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF WHEY PERMEATE

FIELD OF THE INVENTION

The present invention relates to a process for saccharification and fermentation of whey permeate to produce ethanol.

BACKGROUND OF THE INVENTION

Production of ethanol from cheese whey has traditionally been conducted using a yeast belonging to *Kluyveromyces* sp., e.g. *Kluyveromyces fragilis* or *Kluyveromyces marxianus*, since these fermenting organisms can directly ferment lactose to ethanol. However, these yeast suffer from the drawback that they are ethanol sensitive and therefore only processes applying low substrate concentrations and corresponding low ethanol yields have been feasible.

An alternative to using *Kluyveromyces* sp. as the fermenting organism is to use *Saccharomyces cerevisiae*. To make this possible the addition of enzymes, like e.g. a beta-galactosidase, is necessary in order to convert the lactose to fermentable sugars. Mehaia and Cheryan (Bioprocess Engineering 5 (1990), 57-61), describes the production of ethanol from hydrolysed whey permeate using *Saccharomyces cerevisiae* and a beta-galactosidase. In this study the authors obtained an ethanol yield of 0.49 using 10 % dry solids in an SSF process. Because of a high beta-galactosidase concentration conversion of 90% of the lactose was completed in less than one hour.

In SSF hydrolysis/saccharification of lactose is carried out simultaneous with the fermentation and the lactase needs to have sufficient at the pH where the SSF is carried out, typically at a pH between 4.0 and 5.0.

It is the purpose of the present invention to improve ethanol yield of the above *S. cerevisiae* based simultaneous saccharification and fermentation process.

SUMMARY OF THE INVENTION

The invention provides an improved SSF process for producing ethanol from lactose containing substrates.

In a first aspect the present invention relates to a process for producing ethanol from lactose containing substrates, comprising simultaneously saccharifying the substrate to produce monosaccharide and fermenting the monosaccharide to produce ethanol at a pH from 3.5 – 5.5, using a fermenting organism, wherein saccharification is carried out in the presence of a lactase, and wherein the fermenting organism is a *Saccharomyces* sp., and the ratio between the incubation time required for obtaining at least 90% hydrolysis of the lactose present in the substrate (t_1) and the total fermentation time (t_2) is in the range of 0.1 to 1, and the *Saccharomyces* sp. is added in amounts that will result in an ethanol yield of at least 70 % (w/w) of the theoretical ethanol yield from lactose by the end of fermentation.

In a second aspect the present invention relates a process for producing ethanol from starch-containing material comprising the steps of:

- (a) liquefying starch-containing material in the presence of an alpha-amylase; and
- (b) saccharifying the liquefied material obtained in step (a) and a lactose comprising substrate, using at least a glucoamylase and a lactase;
- (c) fermenting using a yeast organism.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows ethanol yield and lactose hydrolysis as a function of fermentation time in SSF run at 32°C, pH 5.0 at 20% DS on a whey permeate substrate using *Bifidobacterium bifidum* lactase (SEQ ID NO: 2) at 0.5 LAU(A)/g DS.

Fig. 2 shows ethanol yield and lactose hydrolysis as a function of fermentation time in SSF run at 32°C, pH 5.0 at 20% DS on a whey permeate substrate using *Bifidobacterium bifidum* lactase (SEQ ID NO: 2) at 1.0 LAU(A)/g DS.

Fig. 3 shows ethanol yield and lactose hydrolysis as a function of fermentation time in SSF run

at 32°C, pH 5.0 at 20% DS on a whey permeate substrate using *Bifidobacterium bifidum* lactase (SEQ ID NO: 2) at 2.0 LAU(A)/g DS.

Fig. 4 shows ethanol yield as a function of fermentation time in SSF run at 32°C, pH 5.0 at 20% DS on a whey permeate substrate using *Bifidobacterium bifidum* lactase (SEQ ID NO: 2) at different lactase concentrations.

Fig. 5 shows ethanol yield and lactose hydrolysis as a function of fermentation time in SSF run at 32°C, pH 5.0 at 20% DS on a whey permeate substrate using *Bifidobacterium bifidum* lactase (SEQ ID NO: 2) at 1.0 LAU(A)/g DS. Included are controls having no lactase enzyme added and SSF performed on 10% galactose as substrate.

Fig. 6 shows kg EtOH/ ton substrate DS as a function of fermentation time wherein EtOH yield was determined by weight loss.

Fig. 7 shows ethanol yield (kg EtOH/ ton substrate DS) as function of fermentation time at three different lactose concentrations.

Fig. 8 shows ethanol yield (kg EtOH/ ton substrate DS) as function of lactase dosage in an SSF-fermentation assay after propagation in glucose + galactose.

Fig. 9 shows ethanol yield (kg EtOH/ ton substrate DS) as function of fermentation time at different %DS and using recycling of the yeast cells.

Fig. 10 shows a comparison of the obtained ethanol yield (kg EtOH/ ton substrate DS) as function of fermentation time using i) propagation in 10% galactose, ii) no propagation, iii) recycling of the yeast.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a process for saccharification and fermentation of a lactose containing substrate to produce ethanol.

More particularly the invention relates to a process for producing ethanol from lactose containing substrates, comprising simultaneously saccharifying the substrate to produce monosaccharide and fermenting the monosaccharide to produce ethanol at a pH from 3.5 – 5.5, using a fermenting organism, wherein saccharification is obtained by the presence of a lactase, and wherein the fermenting organism is a *Saccharomyces* sp., and the ratio between the incubation time required for obtaining at least 90% hydrolysis of the lactose present in the substrate (t_1) and the total fermentation time (t_2) is in the range of 0.1 to 1, and the *Saccharomyces* sp. is added in amounts that will result in an ethanol yield of at least 70% (w/w) of the theoretical ethanol yield from lactose by the end of fermentation.

According to the invention saccharification is obtained in the presence of a lactase (EC 3.2.1.23 and/or EC 3.2.1.108) and the ethanol yield of at least 70 % w/w of the theoretical ethanol yield from lactose (53,8%) can be obtained by adjusting the enzyme dosage and fermentation time to arrive at a suitable t_1/t_2 ratio.

The reaction time necessary for obtaining at least 90% conversion/hydrolysis of the lactose is defined as t_1 and will depend on reaction conditions such as temperature, and pH and on the specific lactase enzyme and enzyme dosage.

In the context of the present invention lactose containing substrates comprise cheese whey, whey, whey permeate, acid whey, permeate mother liquor, and milk. Preferably the substrate is whey permeate or acid whey (e.g., a byproduct of Greek yoghurt).

Lactase enzymes

A lactase in the context of the present invention is any glycoside hydrolase having the ability to hydrolyse the disaccharide lactose into constituent galactose and glucose monomers. The group of lactases comprises but is not limited to enzymes assigned to subclass EC 3.2.1.23. Enzymes assigned to EC 3.2.1.108, may also be lactases in the context of the present invention. A lactase in the context of the invention may have other activities than the lactose hydrolysing activity, such as for example a transgalactosylating activity. In the context of the invention, the lactose hydrolysing activity of the lactase may be referred to as its lactase activity or its beta-galactosidase activity.

Lactase activity according to the present invention may be determined according to the LAU(A) assay described in the examples herein. 1 lactase unit (1 LAU(A)) is the amount of enzyme which releases 1 μ mol glucose per minute in M-buffer at pH 6.5 and 37°C with a lactose concentration of 4.75 % w/v.

Enzymes having lactase activity to be used in a method of the present invention may be of animal, of plant or of microbial origin. Preferred enzymes are obtained from microbial sources, in particular from a filamentous fungus or yeast, or from a bacterium. In particular the lactase enzyme used according to the invention may have optimum activity at a pH range between 3 and 7, preferably from pH 3.5 to 6.0, more preferably pH 4.0 to 5.5, such as around pH 5. The choice of lactase enzyme may depend on the pH of the lactose containing substrate.

The enzyme may, e.g., be derived from a strain of *Agaricus*, e.g. *A. bisporus*; *Ascovaginospora*; *Aspergillus*, e.g. *A. niger*, *A. awamori*, *A. foetidus*, *A. japonicus*, *A. oryzae*; *Candida*; *Chaetomium*; *Chaetotomastia*; *Dictyostelium*, e.g. *D. discoideum*; *Kluveromyces*, e.g. *K. fragilis*, *K. lactis*; *Mucor*, e.g. *M. javanicus*, *M. mucedo*, *M. subtilissimus*; *Neurospora*, e.g. *N. crassa*; *Rhizomucor*, e.g. *R. pusillus*; *Rhizopus*, e.g. *R. arrhizus*, *R. japonicus*, *R. stolonifer*; *Sclerotinia*, e.g. *S. libertiana*; *Torula*; *Torulopsis*; *Trichophyton*, e.g. *T. rubrum*; *Whetzelinia*, e.g. *W. sclerotiorum*; *Bacillus*, e.g. *B. coagulans*, *B. circulans*, *B. megaterium*, *B. novalis*, *B. subtilis*, *B. pumilus*, *B. stearothermophilus*, *B. thuringiensis*; *Bifidobacterium*, e.g. *B. longum*, *B. bifidum*, *B. animalis*; *Chryseobacterium*; *Citrobacter*, e.g. *C. freundii*; *Clostridium*, e.g. *C. perfringens*; *Diplodia*, e.g. *D. gossypina*; *Enterobacter*, e.g. *E. aerogenes*, *E. cloacae* *Edwardsiella*, *E. tarda*; *Erwinia*, e.g. *E. herbicola*; *Escherichia*, e.g. *E. coli*; *Klebsiella*, e.g. *K. pneumoniae*; *Miriococcum*; *Myrothesium*; *Mucor*; *Neurospora*, e.g. *N. crassa*; *Proteus*, e.g. *P. vulgaris*; *Providencia*, e.g. *P. stuartii*; *Pycnopus*, e.g. *Pycnopus cinnabarinus*, *Pycnopus sanguineus*; *Ruminococcus*, e.g. *R. torques*; *Salmonella*, e.g. *S. typhimurium*; *Serratia*, e.g. *S. liquefaciens*, *S. marcescens*; *Shigella*, e.g. *S. flexneri*; *Streptomyces*, e.g. *S. antibioticus*, *S. castaneoglobisporus*, *S. violeceoruber*; *Trametes*; *Trichoderma*, e.g. *T. reesei*, *T. viride*; *Yersinia*, e.g. *Y. enterocolitica*.

In a particular embodiment, the lactase enzyme is from *Aspergillus oryzae*.

In a preferred embodiment, the lactase enzyme is a lactase from a bacterium, e.g. from the

family *Bifidobacteriaceae*, such as from the genus *Bifidobacterium*, such as from a strain of *B. bifidum*, *B. animalis* or *B. longum*. In a more preferred embodiment, the enzyme is a lactase from *Bifidobacterium bifidum*.

In a preferred embodiment, an enzyme having lactase activity to be used in a method of the present invention comprises or consists of the mature polypeptide of SEQ ID NO: 2.

In another preferred embodiment the lactase is selected from the mature polypeptide of SEQ ID NO: 2, or a lactase having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100 % sequence identity to the mature polypeptide of SEQ ID NO: 2.

In a particular embodiment the mature polypeptide is amino acids 28 to 1331 of SEQ ID NO: 2.

In another preferred embodiment the lactase enzyme is obtained from *Aspergillus oryzae*, in particular the lactase is selected from Lactase F (available from Amano) or Tolerase™ L (available from DSM).

Lactase enzyme activity

In the context of the present application, 1 lactase unit (1 LAU(A)) is the amount of enzyme which releases 1 micromole glucose per minute in M-buffer at pH 6.5 and 37°C with a lactose concentration of 4.75% w/v. M-buffer is prepared by dissolving 3.98 g $C_6H_5Na_3O_7 \cdot 2H_2O$, 8.31 g citric acid, 0.9 g K_2SO_4 , 2.6 g K_2HPO_4 , 7.35 g KH_2PO_4 , 5.45 g KOH, 4.15 g $MgCl_2 \cdot 6H_2O$, 3.75 g $CaCl_2 \cdot 2H_2O$ and 1.4 g $NaHCO_3$ in 4 liter water, adding 12.5 ml 4N NaOH, adjusting to pH 6.5 using HCl, and adding water up to a total volume of 5 liter.

The activity in LAU(A) of a specific lactase may be determined by direct measurement of glucose released from lactose under the conditions described above. The skilled person will know how to determine such activity. In particular, the activity may be determined by using the lactase activity assay described in Example 1 of the present application. Here, the activity is obtained by comparing to a standard curve run with a lactase of known activity, and the activity of the unknown sample calculated from this. The lactase of known activity may, e.g., be

LACTOZYM PURE™ obtained from Novozymes A/S, Denmark, and available in different declared activities, e.g., Lactozym Pure 2600 L with a declared activity of 2600 LAU(A)/g.

However, the optimal lactase dosage applied depends on the desired total fermentation time (t_2). The reaction time necessary for obtaining at least 90% conversion/hydrolysis of the lactose is defined herein as t_1 , and will depend on reaction conditions such as temperature, and pH and on the specific lactase enzyme and dosage. In order to obtain a desired ethanol yield of at least 70 % w/w of the theoretical ethanol yield from lactose at the end of fermentation, t_1 and t_2 should according to the invention be adjusted to give t_1/t_2 in the range of 0.1 to 1.0, particularly 0.2 to 1.0, more particularly 0.3 to 1.0, more particularly 0.35 to 0.95.

As described above the process according to the invention relates to a process for producing ethanol from lactose containing substrates, comprising simultaneously saccharifying the substrate to produce monosaccharide and fermenting the monosaccharide to produce ethanol at a pH from 3.5 – 5.5, using a fermenting organism, wherein saccharification is carried out in the presence of a lactase, and wherein the fermenting organism is a *Saccharomyces* sp., and the ratio between the incubation time required for obtaining at least 90% hydrolysis of the lactose present in the substrate (t_1) and the total fermentation time (t_2) is in the range of 0.1 to 1, and the *Saccharomyces* sp. is added in amounts that will result in an ethanol yield of at least 70% w/w of the theoretical ethanol yield from lactose by the end of fermentation.

Optimal conditions will depend on both the hydrolysis time (t_1) and the total fermentation time (t_2). Short total fermentation times will thus also require short hydrolysis time. In order to achieve optimal fermentation conditions the ratio t_1/t_2 should be between 0.1 to 1, more particular 0.2 to 1, particularly 0.3 to 1, and more particular 0.35 to 0.95.

As industrial fermentation times are in the range of 10 to 200 hours the lactase activity should be adjusted in order to provide 90% hydrolysis within this time frame, so that t_1/t_2 is in the range described above.

In one embodiment the lactase activity is adjusted to provide at least 90% hydrolysis of the lactose substrate in 5 to 150 hours, particularly 10 to 130 hours, particularly 30 to 120 hours, more particularly in 40 to 100 hours, more particularly in 50 to 75 hours.

Accordingly in one embodiment fermentation time, t_2 , is in the range from 10-200 hours, particularly 20-150 hours, more particularly 30-130 hours, more particularly 40-120 hours, even

more particularly 50-100 hours.

In the examples this has been illustrated by using a specific lactase enzyme from *Bifidobacterium bifidum*, disclosed herein as the mature polypeptide of SEQ ID NO: 2. Moreover, the yeast pitch was selected to about 30×10^6 , fermentation was performed at pH 5.0 and 32°C, and the fermentation time (t_2) was 150 hours.

It is a particular feature of the invention that the lactase units are adjusted so that the galactose, released from the lactose comprised in the substrate, is made available to the fermenting yeast organism in optimal amounts. It has surprisingly been found that in the case of lactose containing substrates having high dry solids (DS) content, in the present case 12 % DS or more, the lactase activity during the fermentation phase has to be carefully controlled in order to reach the optimal ethanol yields by the end of the fermentation. As shown herein a pre-saccharification of the substrate resulting in complete hydrolysis of the lactose to glucose and galactose does not result in sufficiently high ethanol yields after fermentation. Surprisingly the yeast fermenting organism is only capable of utilizing the glucose but not galactose. Similarly, the galactose cannot be generated too fast, which is the case when the lactase activity is present in amounts of more than 2.0 LAU(A)/g DS using a lactase of SEQ ID NO: 2, at the specified pH, temperature, yeast pitch and fermentation time. It has also been found that galactose can be generated too slowly, which is the case when the lactase activity is present in amounts of less than 0.25 LAU(A)/g DS. The specific values of LAU(A)/g DS may vary depending on the specific lactase applied in the process according to the invention. What seems to be important is controlling the lactase activity in such a way that galactose is provided in optimal amounts.

According to the invention the lactase activity is in the range from 0.05 to 10.0 LAU(A)/g DS when e.g., a lactase from *Bifidobacterium*, in particular *Bifidobacterium bifidum*, is used. More particularly the lactase activity is in the range from 0.1 to 5.0 LAU(A)/g DS, particularly 0.25 to 3.0 LAU(A)/g DS, particularly 0.5 to 2 LAU(A)/g DS, and even more particularly in the range from 0.7 to 1.2 LAU(A)/g DS.

In a further embodiment of the process according to the invention it has been found that it is possible to reduce t_1 , by increasing the LAU(A)/g DS, however this is only possible if the yeast

fermenting organism has been preconditioned to grow in the presence of galactose. According to the invention this preconditioning is termed propagation and means that the yeast fermenting organism, in particular a *Saccharomyces* sp., is cultured in medium comprising galactose prior to the saccharification and fermentation step. Thus in this embodiment of the invention, the *Saccharomyces* sp. is propagated in a solution comprising galactose. In a particular embodiment the solution comprises 1% to 20% galactose, preferably 2% to 15% galactose, more preferably 5% to 10% galactose.

Propagation is at at least 6 hours, particularly at least 12 hours, more particularly at least 24 hours. In a further embodiment propagation is from 6-48 hours, particularly 12-24 hours.

An alternative to propagation would be recycling of the yeast fermenting organism. Thus after fermentation the fermenting organism may be separated from the fermented slurry and recycled.

The process according to the invention comprises hydrolysis (saccharification) and fermentation performed simultaneously (SSF).

When a propagation step is used, lactase activity present during the SSF can be increased and shorter fermentation times are then possible in order to obtain the same ethanol yield. Thus for t_1/t_2 it still applies that t_1/t_2 is in the range from 0.2 to 1, particularly 0.3 to 1, and more particular 0.35 to 0.95. Accordingly in one embodiment the lactase activity is adjusted to provide at least 90% hydrolysis of the lactose substrate in 5 to 100 hours, particularly 10 to 90 hours, particularly 20 to 80 hours, more particularly in 30 to 70 hours. Fermentation times can then also be shortened and thus in a further embodiment the fermentation time is in the range from 10-100 hours, particularly 20-90 hours, more particularly 40-80 hours, more particularly 50-75 hours, even more particularly 60-70 hours.

In one embodiment the lactase activity is in the range from 0.05 to 10.0 LAU(A)/g DS more particularly from 0.1 to 5.0 LAU(A)/g DS, particularly 0.5 to 3.0 LAU(A)/g DS, particularly 1.0 to 2.0 LAU(A)/g DS. The skilled person will know that the specific lactase units needed may depend on the choice of lactase enzyme. In particular the specific ranges disclosed herein have been determined based on the lactase disclosed herein as SEQ ID NO: 2. In this respect also the choice of lactose containing substrate can affect the exact range suitable according to the invention. The substrate pH may e.g., not correspond to the optimal working range for the

lactase enzyme in which case more enzyme activity (e.g., a higher LAU(A)/g DS) has to be added. Thus in a further embodiment the lactase activity is in the range from 2-5 LAU(A)/gDS, more particularly from 3-5 LAU(A)/gDS, even more particularly from 4-5 LAU(A)/gDS.

Dry solids content (DS)

According to the invention the DS should preferably be at least 12%, such as least 13%, more particularly at least 14%, more particularly at least 15%, and even more particularly at least 20%. In one embodiment the DS is in the range from 12-40%, particularly from 15-30%, more particularly from 20-25%.

Fermenting organism

In one embodiment the fermenting organism, the *Saccharomyces* sp., is added to the fermentation medium so that the viable count per mL of fermentation medium (yeast pitch) is in the range from 10^5 to 10^{12} , preferably from 10^6 to 10^8 , such as around 10^7 , especially about 30×10^6 .

Yeast is the preferred fermenting organism for ethanol fermentation. Preferred are strains of *Saccharomyces*, especially strains of the species *Saccharomyces cerevisiae*, preferably strains which are resistant towards high levels of ethanol, i.e., up to, e.g., about 10, 12, 15 or 20 vol. % or more ethanol.

Commercially available yeast include LNF SA1, LNF BG1, LNF PE2, and LNF CAT1 (available from LNF Brazil), RED STAR™ and 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), and FERMIOL (available from DSM Specialties).

In a further embodiment it is contemplated that the fermenting yeast is expressing the lactase in suitable amounts in order to provide a t_1/t_2 ratio in the range discussed herein. The skilled

person will know how to regulate gene expression in yeast.

Fermentation

The fermentation conditions are determined based on, *e.g.*, the kind of plant material, the available fermentable sugars, the fermenting organism(s) and/or the desired fermentation product. One skilled in the art can easily determine suitable fermentation conditions. The fermentation may be carried out at conventionally used conditions. Preferred fermentation processes are anaerobic processes.

For ethanol production using yeast, the fermentation is carried out at a temperature between 20 to 40°C, preferably 28 to 36°C, in particular around 32°C. In an embodiment the pH is from pH 3 to 6, particularly pH from 3.5 to 5.5, preferably around pH 5.

Especially contemplated is simultaneous hydrolysis/saccharification and fermentation (referred to as "SSF") where there is no separate holding stage for the hydrolysis/saccharification, meaning that the hydrolysing enzyme(s), and the fermenting organism(s), are added together. When fermentation is performed simultaneous with saccharification (*i.e.*, SSF) the temperature is preferably between 20 to 40°C, preferably 28 to 36°C, in particular around 32°C when the fermentation organism is a strain of *Saccharomyces cerevisiae* and the desired fermentation product is ethanol.

In a particular embodiment the fermentation time (t_2) is at least 10 hours, more particularly at least 20 hours, more particularly at least 30 hours, more particularly at least 40 hours, more particularly at least 50 hours, more particularly at least 60 hours, more particularly at least 70 hours, more particularly at least 80 hours, more particularly at least 90 hours, more particularly at least 100 hours, more particularly at least 110 hours, more particularly at least 120 hours, more particularly at least 130 hours, more particularly at least 140 hours, more particularly at least 150 hours.

The total fermentation time is defined as t_2 . In the case of continuous or fed-batch fermentations t_2 is defined as the mean residence time of the yeast in the fermenter.

Conventional Starch Processing

Native starch consists of microscopic granules, which are insoluble in water at room

temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. At temperatures up to about 50°C to 75°C the swelling may be reversible. However, with higher temperatures an irreversible swelling called "gelatinization" begins. During this "gelatinization" process there is a dramatic increase in viscosity. Granular starch to be processed may be a highly refined starch quality, preferably at least 90%, at least 95%, at least 97% or at least 99.5% pure or it may be a more crude starch-containing materials comprising (e.g., milled) whole grains including non-starch fractions such as germ residues and fibers. The raw material, such as whole grains, may be reduced in particle size, e.g., by milling, in order to open up the structure and allowing for further processing. In dry milling whole kernels are milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is often applied at locations where the starch hydrolyzate is used in the production of, e.g., syrups. Both dry and wet milling are well known in the art of starch processing and may be used in a process of the invention. Methods for reducing the particle size of the starch containing material are well known to those skilled in the art.

As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be suitably processed. This reduction in viscosity is primarily attained by enzymatic degradation in current commercial practice.

Liquefaction is carried out in the presence of an alpha-amylase, preferably a bacterial alpha-amylase and/or acid fungal alpha-amylase. In an embodiment, a phytase is also present during liquefaction. In an embodiment, viscosity reducing enzymes such as a xylanase and/or beta-glucanase is also present during liquefaction.

During liquefaction, the long-chained starch is degraded into branched and linear shorter units (maltodextrins) by an alpha-amylase. Liquefaction may be carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C (e.g., 70-90°C, such as 77-86°C, 80-85°C, 83-85°C) and an alpha-amylase is added to initiate liquefaction (thinning).

The slurry may in an embodiment be jet-cooked at between 95-140°C, e.g., 105-125°C, for about 1-15 minutes, e.g., about 3-10 minutes, especially around 5 minutes. The slurry is then cooled to 60-95°C and more alpha-amylase is added to obtain final hydrolysis (secondary liquefaction). The jet-cooking process is carried out at pH 4.5-6.5, typically at a pH between 5 and 6. The alpha-amylase may be added as a single dose, e.g., before jet cooking.

The liquefaction process is carried out at between 70-95°C, such as 80-90°C, such as around 85°C, for about 10 minutes to 5 hours, typically for 1-2 hours. The pH is between 4 and 7, such as between 5.5 and 6.2. In order to ensure optimal enzyme stability under these conditions, calcium may optionally be added (to provide 1-60 ppm free calcium ions, such as about 40 ppm free calcium ions). After such treatment, the liquefied starch will typically have a "dextrose equivalent" (DE) of 10-15.

Generally liquefaction and liquefaction conditions are well known in the art.

Saccharification may be carried out using conditions well known in the art with a carbohydrate-source generating enzyme, in particular a glucoamylase, or a beta-amylase and optionally a debranching enzyme, such as an isoamylase or a pullulanase. For instance, a full saccharification step may last from about 24 to about 72 hours. However, it is common to do a pre-saccharification of typically 40-90 minutes at a temperature between 30-65°C, typically about 60°C, followed by complete saccharification during fermentation in a simultaneous saccharification and fermentation (SSF) process. Saccharification is typically carried out at a temperature in the range of 20-75°C, *e.g.*, 25-65°C and 40-70°C, typically around 60°C, and at a pH between about 4 and 5, normally at about pH 4.5.

The saccharification and fermentation steps may be carried out either sequentially or simultaneously. In an embodiment, saccharification and fermentation are performed simultaneously (referred to as "SSF"). However, it is common to perform a pre-saccharification step for about 30 minutes to 2 hours (*e.g.*, 30 to 90 minutes) at a temperature of 30 to 65°C, typically around 60°C which is followed by a complete saccharification during fermentation referred to as simultaneous saccharification and fermentation (SSF).

The above described conventional starch ethanol process may in one embodiment be combined with the process according to the present invention. In this particular embodiment the present invention relates to a process for producing ethanol from starch-containing material comprising the steps of:

- (a) liquefying starch-containing material in the presence of an alpha-amylase; and
- (b) saccharifying the liquefied material obtained in step (a) and a lactose comprising substrate, using at least a glucoamylase and a lactase;

(c) fermenting using a yeast organism.

In particular, saccharification and fermentation are performed simultaneously (referred to as "SSF"). The lactose comprising substrate is in a particular embodiment whey permeate, acid whey, or milk.

Usually the mash is cooled after the liquefaction step and in a preferred embodiment the lactose substrate and/or the lactase is added to the mash during or after cooling or in a further embodiment the lactose substrate and/or the lactase is added prior to or during fermentation.

The present invention is further described by the following numbered paragraphs:

Paragraph [1]. A process for producing ethanol from lactose containing substrates, comprising simultaneously saccharifying the substrate to produce monosaccharide and fermenting the monosaccharide to produce ethanol at a pH from 3.5 – 5.5, using a fermenting organism, wherein saccharification is carried out in the presence of a lactase, and wherein the fermenting organism is a *Saccharomyces* sp., and the ratio between the incubation time required for obtaining at least 90% hydrolysis of the lactose present in the substrate (t_1) and the total fermentation time (t_2) is in the range of 0.1 to 1, and the *Saccharomyces* sp. is added in amounts that will result in an ethanol yield of at least 70 % w/w of the theoretical ethanol yield from lactose by the end of fermentation.

Paragraph [2]. The process according to paragraph 1, wherein t_1/t_2 is in the range from 0.2 to 1, particularly 0.3 to 1, and more particular 0.35 to 0.95.

Paragraph [3]. The process according to paragraph 1-2, wherein the lactase activity is adjusted to provide at least 90% hydrolysis of the lactose substrate in 5 to 150 hours, particularly 10 to 130 hours, particularly 30 to 120 hours, more particularly in 40 to 100 hours, more particularly in 50 to 75 hours.

Paragraph [4]. The process according to any of the paragraphs 1-3, wherein the fermentation time is in the range from 10-200 hours, particularly 20-150 hours, more particularly 30-130

hours, more particularly 40-120 hours, even more particularly 50-100 hours.

Paragraph [5]. The process according to paragraph 1, wherein the lactase activity is in the range from 0.05 to 10.0 LAU(A)/g DS more particularly from 0.1 to 5.0 LAU(A)/g DS, particularly 0.25 to 3.0 LAU(A)/g DS, particularly 0.5 to 2 LAU(A)/g DS, and even more particularly in the range from 0.7 to 1.2 LAU(A)/g DS.

Paragraph [6]. The process according to paragraph 1, wherein the *Saccharomyces* sp. fermenting organism is propagated in a solution comprising galactose.

Paragraph [7]. The process according to paragraph 6, wherein the solution comprises 1% to 20% galactose, preferably 2% to 15% galactose, 5% to 10% galactose.

Paragraph [8]. The process according to paragraphs 6-7, wherein preincubation is at least 6 hours, particularly at least 12 hours, more particularly at least 24 hours.

Paragraph [9]. The process according to paragraphs 6-8, wherein the lactase activity is adjusted to provide at least 90% hydrolysis of the lactose substrate in 5 to 100 hours, particularly 10 to 90 hours, particularly 20 to 80 hours, more particularly in 30 to 70 hours.

Paragraph [10]. The process according to any of the paragraphs 6-9, wherein the fermentation time is in the range from 10-100 hours, particularly 20-90 hours, more particularly 40-80 hours, more particularly 50-75 hours, even more particularly 60-70 hours.

Paragraph [11]. The process according to any paragraphs 6-10, wherein the lactase activity is in the range from 0.05 to 10.0 LAU(A)/g DS more particularly from 0.1 to 5.0 LAU(A)/g DS, particularly 0.5 to 3.0 LAU(A)/g DS, particularly 1.0 to 2.0 LAU(A)/g DS.

Paragraph [12]. The process according to any of paragraphs 1-11, wherein the *Saccharomyces* sp. is *Saccharomyces cerevisiae*.

Paragraph [13]. The process according to any of the preceding paragraphs, wherein the Dry solid content, DS, is at least 12%, more particularly at least 13%, more particularly at least 14%, more particularly at least 15%, even more particularly at least 20%.

Paragraph [14]. The process according to any of the preceding paragraphs, wherein the lactose containing substrate is whey permeate, acid whey, or milk.

Paragraph [15]. The process according to any of the preceding paragraphs, wherein the lactase enzyme is obtained from a strain of *Bifidobacterium*, or from *Aspergillus*.

Paragraph [16]. The process according to paragraph 15, wherein the lactase enzyme is obtained from *Bifidobacterium bifidum*, or *Aspergillus oryzae*.

Paragraph [17]. The process according to any of the paragraphs 1-16, wherein the lactase is selected from the mature polypeptide of SEQ ID NO: 2, or a lactase having at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100 % sequence identity to the mature polypeptide of SEQ ID NO: 2.

Paragraph [18]. The process according to paragraph 17, wherein the mature polypeptide is amino acids 28 to 1331 of SEQ ID NO: 2.

Paragraph [19]. The process according to any of the paragraphs 1-18, wherein the lactase enzyme activity is provided by the yeast fermenting organism, expressing and secreting the lactase enzyme in suitable amounts.

Paragraph [20]. The process according to any of the preceding paragraphs wherein the yeast pitch is 10^6 - 10^8 , such as around 10^7 viable count/ml.

Paragraph [21]. A process for producing ethanol from starch-containing material comprising the steps of:

- (a) liquefying starch-containing material in the presence of an alpha-amylase; and

- (b) saccharifying the liquefied material obtained in step (a) and a lactose comprising substrate, using at least a glucoamylase and a lactase;
- (c) fermenting using a yeast organism.

Paragraph [22]. The process according to paragraph 21, wherein saccharification and fermentation are performed simultaneously.

Paragraph [23]. The process according to any of paragraphs 21-22, wherein the lactose comprising substrate is whey permeate, acid whey, or milk.

Paragraph [24]. The process according to any of paragraphs 21-23, further comprising the steps of:

- a) cooling the liquefied substrate (mash) after the liquefaction step; and
- b) adding a lactose substrate and the lactase to the mash during or after cooling.

Paragraph [25]. The process according to paragraph 21, wherein the lactose substrate and the lactase is added prior to or during fermentation.

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

Example 1. Determination of lactase activity (LAU(A))

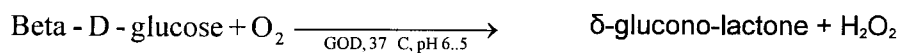
Principle:

Lactase hydrolyses lactose into glucose and galactose. Glucose is measured after a modified version of the common glucose oxidase / peroxidase assay (Werner, W. *et al.* (1970) *Z. analyt. Chem.* **252**: 224.).

The amount of glucose formed is determined using the GOD-Perid method:

Glucose-oxidase (β -D-glucose: oxygen-1-oxido-reductase, EC 1.1.3.4.) oxidises β -D-glucose

in the presence of oxygen to δ -glucono-lactone and hydrogen-peroxide. The generated hydrogen-peroxide oxidises ABTS-R (2, 2-Azino-di-(3-ethylbenzthiazoline)-6-sulfonate) in the presence of peroxidase (POD). This generates a green-blue colour, which is measured photometrically at 675 nm.



Parameter	Reaction conditions
Temperature	37°C
pH	6.5
Substrate conc.	50 g/L
Enzyme conc.	0,0117-0,0333 LAU/mL
Reaction time	30 min.
Wave length	675 nm

1 lactase unit (1 LAU(A)) is the amount of enzyme which releases 1 μmol glucose per minute in M-buffer at pH 6.5 and 37°C with a lactose concentration of 4.75 % w/v.

The detailed method is available from Novozymes A/S on request.

Example 2. Determination of ethanol yield as function of lactase dosage in an SSF-fermentation assay.

The *Bifidobacterium bifidum* lactase disclosed in SEQ ID NO: 2 was tested in the process according to the invention. This lactase was used for all examples included.

Fermentation assay:

Prepare 1L 20% w/w whey permeate substrate (e.g. use Variolac 836 from Arla Foods).

Add urea (800 ppm) and adjust pH to pH 5.0

Add penicillin G (1 ppm) and yeast (Red Ethanol 30 10⁶/ ml)

Empty flask are weighed in advance

75 g substrate is transferred to 250 ml fermentation shaking flasks

Add lactase according to schedule

Flasks are weighed again, t=0

The fermentation flasks are placed in the shaking incubator at 32 °C (80 rpm)

Measure weight loss twice a day

HPLC measurement after 48, 72, and 144 hours (inactivate enzyme with HCl immediately after taking the sample)

Substrate:

Variolac® 836 from Arla Foods Ingredients

Chemical specifications:

Protein (Nx6,38) as is	max. 5.0%
Lactose monohydrate	min. 83.0%
Ash	max. 10.0%
Moisture	Max. 3.0%

The maltose content in Variolac 836 was determined to 87 % by HPLC.

HPLC analytic method:

Two columns in series: BIO-RAD Aminex HPC-87H, cat no 128-014

Solvent : 0,005M H₂SO₄

Retention times (minutes) are:

Lactose= 23.6; glucose= 27.8; galactose= 29.5

Table 1. The table shows ethanol yields obtained in SSF using different enzyme doses.

Enzyme dose LAU(A)/ g DS (SEQ ID NO: 2)	Time (SSF) Hours	Lactose g/l	Glucose g/l	Galactose g/l	Ethanol Kg /t substrate DS
0.5	0	170	0	0	0
0.5	48	90.04	0.26	1.45	168.3
0.5	72	49.37	0.29	2.03	241.6
0.5	144	5.87	0.40	2.16	347.3
1.0	0	170	0	0	0
1.0	48	31.13	0.42	13.77	248.9
1.0	72	5.92	0.63	21.00	292.7
1.0	144	1.04	0.84	17.24	320.6
2.0	0	170	0	0	0
2.0	48	7.11	7.11	57.35	208.9
2.0	72	4.17	4.17	55.96	213.3
2.0	144	2.41	2.41	45.43	226.2

The data from the table was plotted in Figures 1, 2 and 3.

100% conversion of the substrate corresponds to approximately 450 kg EtOH/ t substrate DS.

The fermentation times resulting in a remaining lactose concentration of 10% (90% hydrolysis) can be read from the above graphs and are:

Table 2.

Enzyme dose	Time to 90% hydrolysis (hours)	t_1/t_2
0.5 LAU(A)/ g DS	130	0.9
1.0 LAU(A)/ g DS	60	0.42
2.0 LAU(A)/ g DS	45	0.31

Optimal performance was observed when the time to 90% hydrolysis was close to the total fermentation time and thus that the sugars were made available to the yeast simultaneously with the ethanol production.

Example 3. Determination of ethanol yield as function of higher lactase dosages in an SSF-fermentation assay.

The effect of increasing the enzyme dosage, *Bifidobacterium bifidum* lactase disclosed in SEQ ID NO: 2, was tested in a similar setup as described in example 2. Fermentation assay, substrate and HPLC were unchanged.

The results are shown in table 3 below and in figure 4.

Table 3. HPLC-data after 72 hours fermentation time:

Enzyme dose	Lactose g/l	Glucose g/l	Galactose g/l	EtOH g/l	EtOH Kg/ t substrate

					NB: based on weight loss
1 LAU(A)/g	6.40	0.49	6.07	66.15	332.9
5 LAU(A)/g	4.11	0.57	73.74	39.66	198
5 LAU(A)/g	4.11	0.55	75.31	39.49	
10 LAU(A)/g	3.11	0.4	83.62	38.18	193
10 LAU(A)/g	3.09	0.4	83.66	38.3	
20 LAU(A)/g	3.04	0.43	83.58	38.15	194
20 LAU(A)/g	3	0.43	83.32	37.89	

It is concluded from Example 3 that the yeast is not able to ferment galactose when the high LAU(A)-dosages are used in the SSF-fermentation even though the lactose is almost completely hydrolyzed.

Example 4: Determination of ethanol yield in an SSF-fermentation assay. Control experiments with galactose and whey permeate without addition of enzyme

This example illustrates the effects of not adding any lactase enzyme to the whey permeate substrate or using a substrate containing only galactose (10% galactose).

Fermentation assay, Substrate, and HPLC-method as in Example 2 except that the substrate concentration was only 10% in the test with pure galactose. The result is shown in table 4 and in fig. 5.

Table 4. HPLC-data for the test with 10% galactose:

	Lactose g/l	Glucose g/l	Galactose g/l	EtOH g/l
Galactose (10% DS)	3.56	0.07	72.49	9.86

The results show that without adding any lactase to the SSF process the *S. cerevisiae* fermenting organism is not capable of fermenting the lactose substrate, and that *S. cerevisiae* can only ferment pure galactose very slowly.

Example 5. Determination of ethanol yield in an SSF-fermentation with prehydrolyzed whey permeate

Preparation of substrate:

A 25% DS solution was made from whey permeate (Variolac 836) and DI water and incubated with Lactase added at a dosage of 20 LAU(A)/g DS. pH was adjusted to 5.0.

The solution is incubated at 32°C over-night and then inactivated (boiled) for 10 minutes. After cooling the substrate is ready for fermentation. Samples were taken for HPLC analysis.

Fermentation assay and HPLC-method as in Example 2.

Table 5. HPLC-data:

Time (SSF) Hours	Lactose g/l	Glucose g/l	Galactose g/l	Ethanol g/l
0	3.2	110.3	112.8	0.9

48	2.9	0.2	94.8	46.1
72	5.8	0.2	91.0	49.5
144	2.7	0.4	83.4	53.3

It is concluded that yeast can ferment glucose, but only to a minor extent galactose when the lactose is hydrolyzed before fermentation.

Example 6: Determination of ethanol yield as function of lactase dosage in an SSF-fermentation assay after propagation in galactose.

Fermentation assay, substrate, and HPLC-method as in Example 2 except that the yeast was propagated in galactose.

The procedure for yeast propagation was:

The yeast was taken out of the fridge and suspended in demineralized water. Galactose was added to a concentration of 10% w/w and agitated for 24 hours at room temperature before use. Ethanol yield data is shown in table 6 below and in fig. 7.

Table 6. HPLC data (after 72 hours):

	Lactose g/l	Glucose g/l	Galactose g/l	EtOH g/l
0.5 LAU(A)/ g DS	70.2	0.3	0.5	44.5
1.0 LAU(A)/ g DS	0.9	0.7	0.1	78.6
2.0 LAU(A) / g DS	0.6	0.6	0.1	79.8

Example 7: Determination of ethanol yield as function of lactase dosage in an SSF-fermentation assay after propagation in glucose + galactose.

Fermentation assay, substrate, and HPLC-method as in Example 2 except that the yeast was propagated in mixture of 10% glucose and 10% galactose.

The procedure for yeast propagation was:

The yeast was taken out of the fridge and suspended in demineralized water. Glucose and galactose was added to a concentration of 10% w/w each and agitated for 24 hours before use. Ethanol yield data is shown in table 7 and in fig. 8. The results obtained were identical when the same lactase concentration was used with or without glucose present. Therefore the resulting curves cannot be distinguished.

Table 7. HPLC data (after 116 hours):

Sample	EtOH (g/l)
0.5 LAU(A)/ g DS; propagated in 10% galactose	65.4
0.5 LAU(A)/ g DS; propagated in 10% galactose + 10% glucose	67.4
1 LAU(A)/ g DS; propagated in 10% galactose	79.7
1 LAU(A)/ g DS; propagated in 10% galactose + 10% glucose	80.0
2 LAU(A)/ g DS; propagated in 10% galactose	82.0
2 LAU(A)/ g DS; propagated in 10% galactose + 10% glucose	82.9

The propagation in 10% glucose and 10% galactose simulates propagation in a 20% lactose

solution hydrolyzed with lactase. The result showed that presence of glucose did not have an effect.

Example 8: Determination of ethanol yield with recycling of the yeast

Fermentation assay, substrate, and HPLC-method as in Example 2 except that the yeast was recycled from a previous fermentation.

The procedure for yeast recycle was:

The yeast from Example 7, the test with propagation in 10% galactose, was split in two equal parts after the fermentation and used as the recycled yeast in this experiment.

The result is shown in fig. 9 in which the ethanol yield is plotted as a function of fermentation time. The end final ethanol yield was about the same level for both tested dry solid concentrations. Compared to data obtained by not using recycling (see example 2, table 1 and fig. 3) the results show that higher ethanol yield can be reached by recycling the yeast compared to adding the lactose during the SSF step (0.5-1.0 LAU(A) was the optimal lactose concentration under the conditions in example 2).

Data in fig. 10 illustrates the optimal ethanol yields obtainable using three different embodiments of the process according to the invention. Embodiment 1 is adding the lactase to the SSF step in the optimal dosage range (about 0.5 LAU(A)/g DS). Embodiment 2 includes recycling of the yeast and adding lactose in the optimal dosage range (about 2.0 LAU(A)/g DS). Embodiment 3 includes propagation of the yeast in 10% galactose and 10% glucose (simulating a 20% whey permeate substrate) prior to SSF and using the same lactose dosage as embodiment 2.

The data are from example 2, 7, and 8 and the results show that the highest possible ethanol yield was reached using embodiment 3 (propagation of the yeast) followed by embodiment 2 (recycling of the yeast).

CLAIMS

1. A process for producing ethanol from lactose containing substrates, comprising simultaneously saccharifying the substrate to produce monosaccharide and fermenting the monosaccharide to produce ethanol at a pH from 3.5 – 5.5, using a fermenting organism, wherein saccharification is carried out in the presence of a lactase, and wherein the fermenting organism is a *Saccharomyces* sp., and the ratio between the incubation time required for obtaining at least 90% hydrolysis of the lactose present in the substrate (t_1) and the total fermentation time (t_2) is in the range of 0.1 to 1, wherein the fermentation time is in the range from 20-150 hours and the lactase activity is added in a range from 0.1 to 5.0 LAU(A)/g Dry Solid Content (DS) and the *Saccharomyces* sp. is added in amounts that will result in an ethanol yield of at least 70 % w/w of the theoretical ethanol yield from lactose by the end of fermentation.
2. The process according to claim 1, wherein t_1/t_2 is in the range from 0.2 to 1.
3. The process according to claim 1, wherein t_1/t_2 is in the range from 0.3 to 1.
4. The process according to claim 1, wherein t_1/t_2 is in the range from 0.35 to 0.95.
5. The process according to any one of claims 1 to 4, wherein the lactase activity is adjusted to provide at least 90% hydrolysis of the lactose substrate in 30 to 120 hours.
6. The process according to any one of claims 1 to 4, wherein the lactase activity is adjusted to provide at least 90% hydrolysis of the lactose substrate in 40 to 100 hours.
7. The process according to any one of claims 1 to 4, wherein the lactase activity is adjusted to provide at least 90% hydrolysis of the lactose substrate in 50 to 75 hours.
8. The process according to any one of claims 1 to 4, wherein the fermentation time is in the range from 30-130 hours.

9. The process according to any one of claims 1 to 4, wherein the fermentation time is in the range from 40-120 hours.
10. The process according to any one of claims 1 to 4, wherein the fermentation time is in the range from 50-100 hours.
11. The process according to claim 1, wherein the lactase activity is in the range from 0.25 to 3.0 LAU(A)/g DS.
12. The process according to claim 1, wherein the lactase activity is in the range from 0.5 to 2 LAU(A)/g DS.
13. The process according to claim 1, wherein the lactase activity is in the range from 0.7 to 1.2 LAU(A)/g DS.
14. The process according to claim 1, wherein the *Saccharomyces* sp. fermenting organism is propagated in a solution comprising galactose.
15. The process according to claim 14, wherein the solution comprises 1% to 20% galactose.
16. The process according to claim 14, wherein the solution comprises 2% to 15% galactose.
17. The process according to claim 14, wherein the solution comprises 5% to 10% galactose.
18. The process according to any one of claims 14 to 17, wherein the *Saccharomyces* sp. is propagated at least 6 hours.
19. The process according to any one of claims 14 to 17, wherein the *Saccharomyces* sp. is propagated at least 12 hours.
20. The process according to any one of claims 14 to 17, wherein the *Saccharomyces* sp. is propagated at least 24 hours.

21. The process according to any one of claims 14 to 20, wherein the lactase activity is adjusted to provide at least 90% hydrolysis of the lactose substrate in 20 to 80 hours.
22. The process according to any one of claims 14 to 20, wherein the lactase activity is adjusted to provide at least 90% hydrolysis of the lactose substrate in 30 to 70 hours.
23. The process according to any one of claims 14 to 20, wherein the fermentation time is in the range from 20-90 hours.
24. The process according to any one of claims 14 to 20, wherein the fermentation time is in the range from 40-80 hours.
25. The process according to any one of claims 14 to 20, wherein the fermentation time is in the range from 50-75 hours.
26. The process according to any one of claims 14 to 20, wherein the fermentation time is in the range from 60-70 hours.
27. The process according to any one of claims 14 to 26, wherein the lactase activity is in the range from 0.5 to 3.0 LAU(A)/g DS.
28. The process according to any one of claims 14 to 26, wherein the lactase activity is in the range from 1.0 to 2.0 LAU(A)/g DS.
29. The process according to any one of claims 1 to 28, wherein the *Saccharomyces* sp. is *Saccharomyces cerevisiae*.
30. The process according to any one of claims 1 to 29, wherein the Dry solid content, DS, is at least 12%.
31. The process according to any one of claims 1 to 29, wherein the Dry solid content, DS, is

at least 13%.

32. The process according to any one of claims 1 to 29, wherein the Dry solid content, DS, is at least 14%.

33. The process according to any one of claims 1 to 29, wherein the Dry solid content, DS, is at least 15%.

34. The process according to any one of claims 1 to 29, wherein the Dry solid content, DS, is at least 20%.

35. The process according to any one of claims 1 to 34, wherein the lactose containing substrate is whey permeate, acid whey, or milk.

36. The process according to any one of claims 1 to 35, wherein the lactase enzyme is obtained from a strain of *Bifidobacterium*, or from *Aspergillus*.

37. The process according to claim 36, wherein the lactase enzyme is obtained from *Bifidobacterium bifidum*, or *Aspergillus oryzae*.

38. The process according to any one of claims 1 to 37, wherein the lactase has at least 90% sequence identity to the mature polypeptide of SEQ ID NO: 2.

39. The process according to any one of claims 1 to 37, wherein the lactase has at least 95% sequence identity to the mature polypeptide of SEQ ID NO: 2.

40. The process according to any one of claims 1 to 37, wherein the lactase has at least 96% sequence identity to the mature polypeptide of SEQ ID NO: 2.

41. The process according to any one of claims 1 to 37, wherein the lactase has at least 97% sequence identity to the mature polypeptide of SEQ ID NO: 2.

42. The process according to any one of claims 1 to 37, wherein the lactase has at least 98% sequence identity to the mature polypeptide of SEQ ID NO: 2.
43. The process according to any one of claims 1 to 37, wherein the lactase has at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 2.
44. The process according to any one of claims 1 to 37, wherein the lactase has 100% sequence identity to the mature polypeptide of SEQ ID NO: 2.
45. The process according to any one of claims 38 to 44, wherein the mature polypeptide is amino acids 28 to 1331 of SEQ ID NO: 2.
46. The process according to any one of claims 1 to 45, wherein the lactase enzyme activity is provided by the *Saccharomyces* sp., expressing and secreting the lactase enzyme in suitable amounts.
47. The process according to any of claims 1 to 46, wherein the *Saccharomyces* sp. is added in amounts that result in a yeast pitch of 10^6 - 10^8 viable count/ml.
48. The process according to any of claims 1 to 46, wherein the *Saccharomyces* sp. is added in amounts that result in a yeast pitch of 10^7 viable count/ml.

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Figure 1.

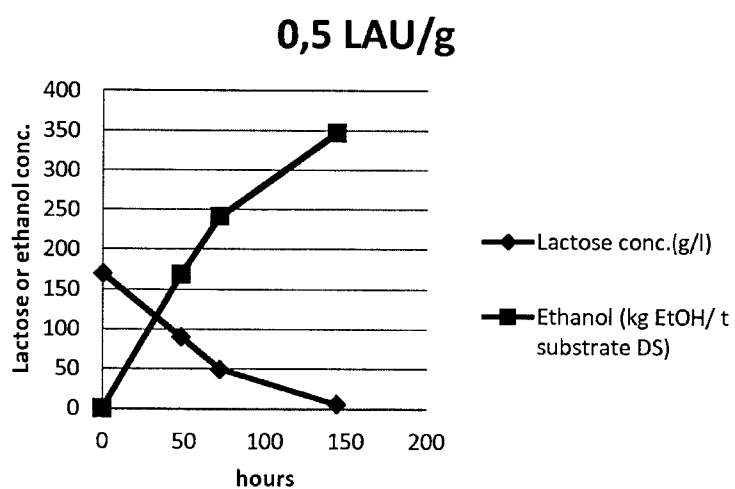
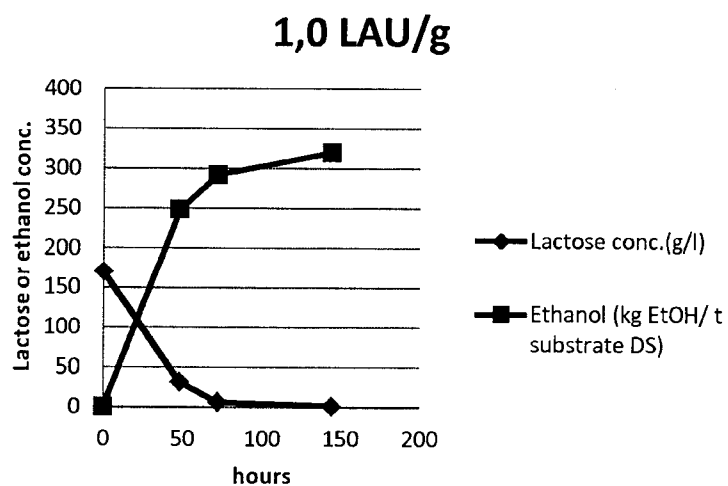


Figure 2.



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Figure 3.

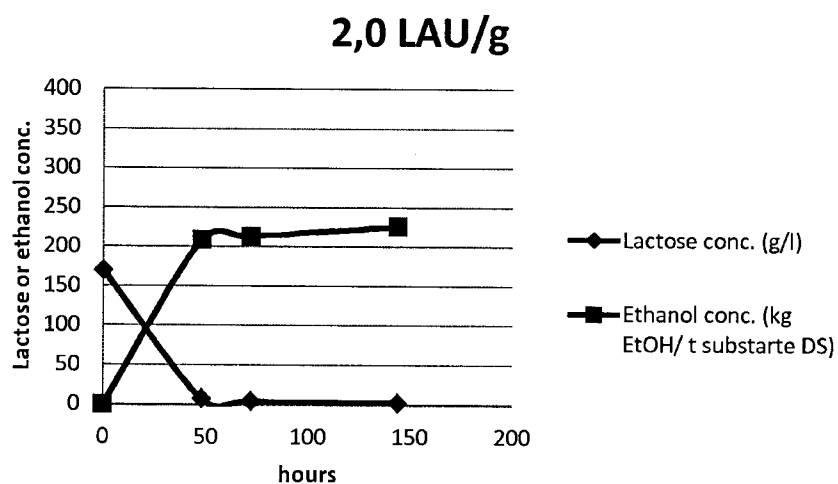
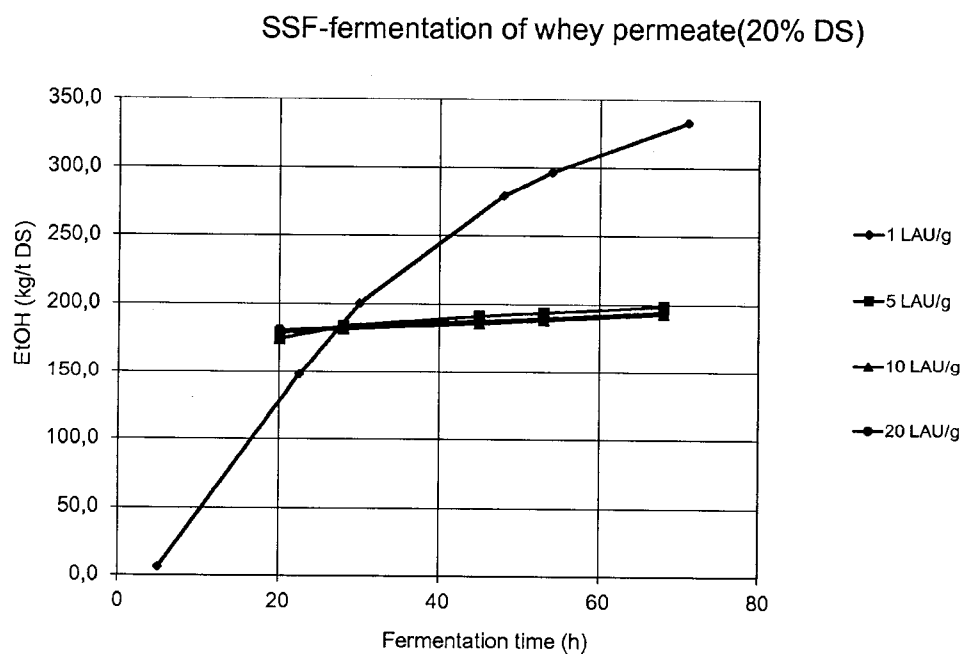


Figure 4.



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Figure 5.

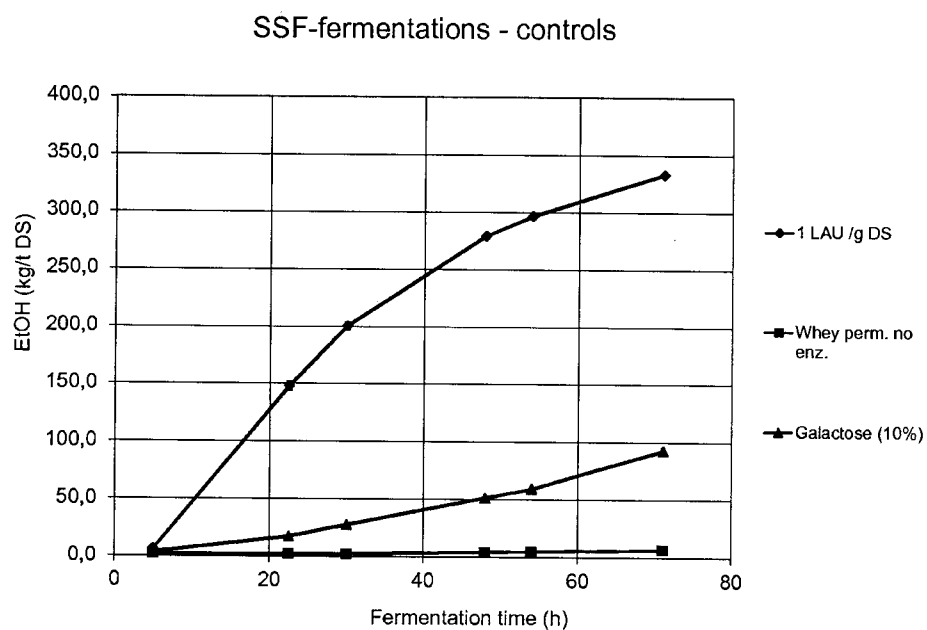
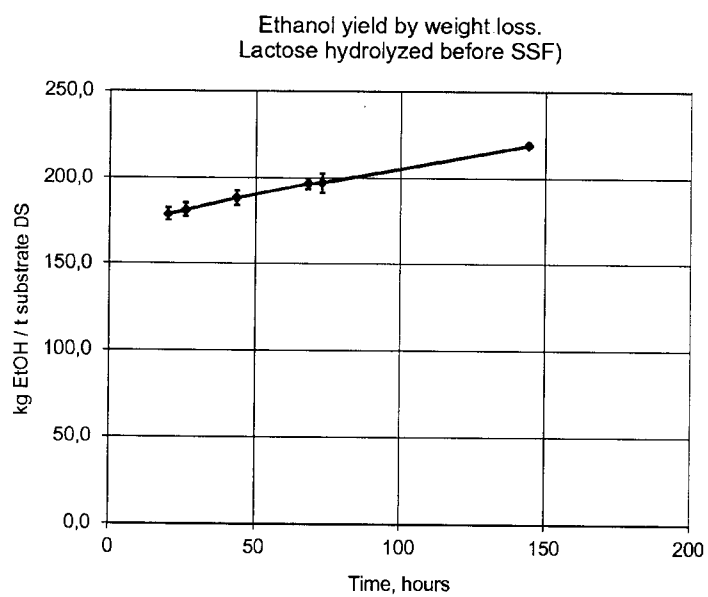


Figure 6.



SUBSTITUTE SHEET (RULE 26)

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Figure 7.

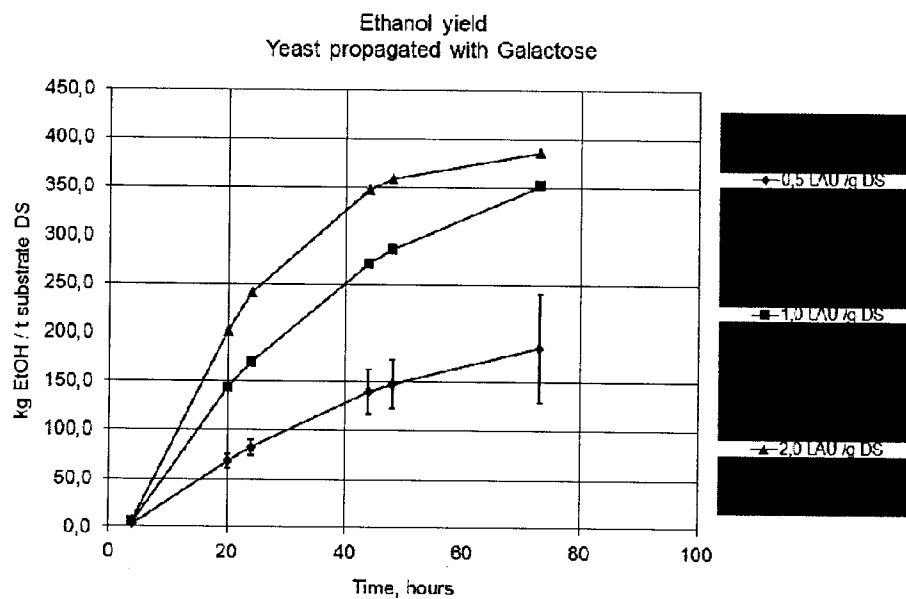
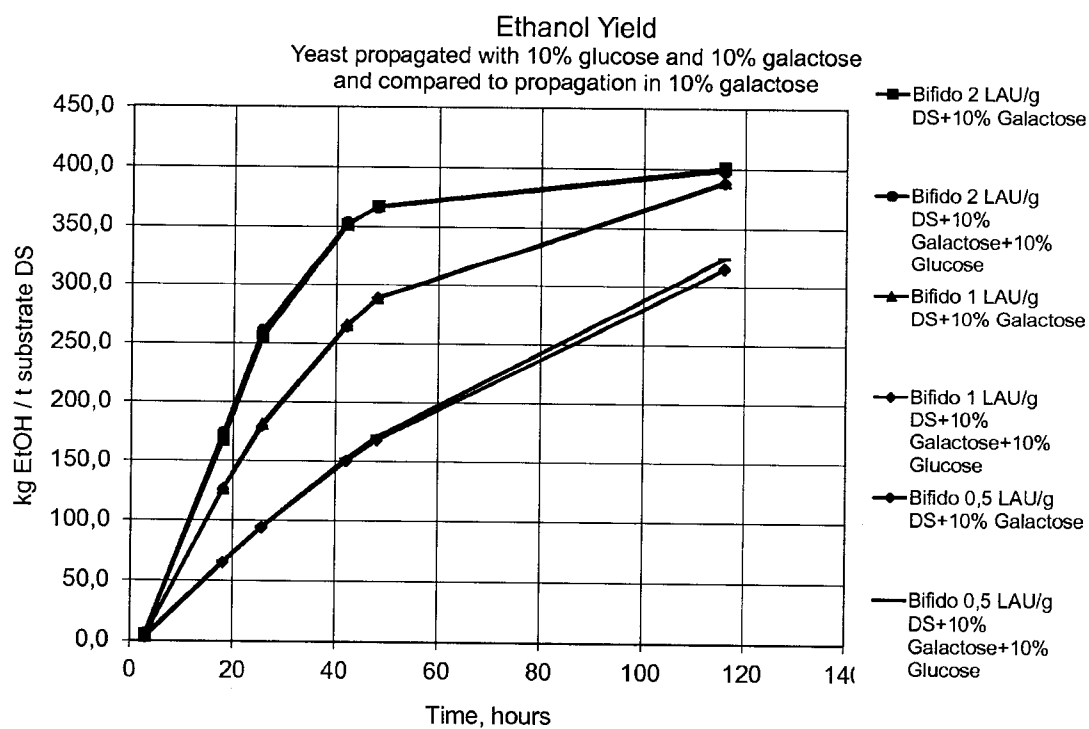


Figure 8.



SUBSTITUTE SHEET (RULE 26)

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Figure 9.

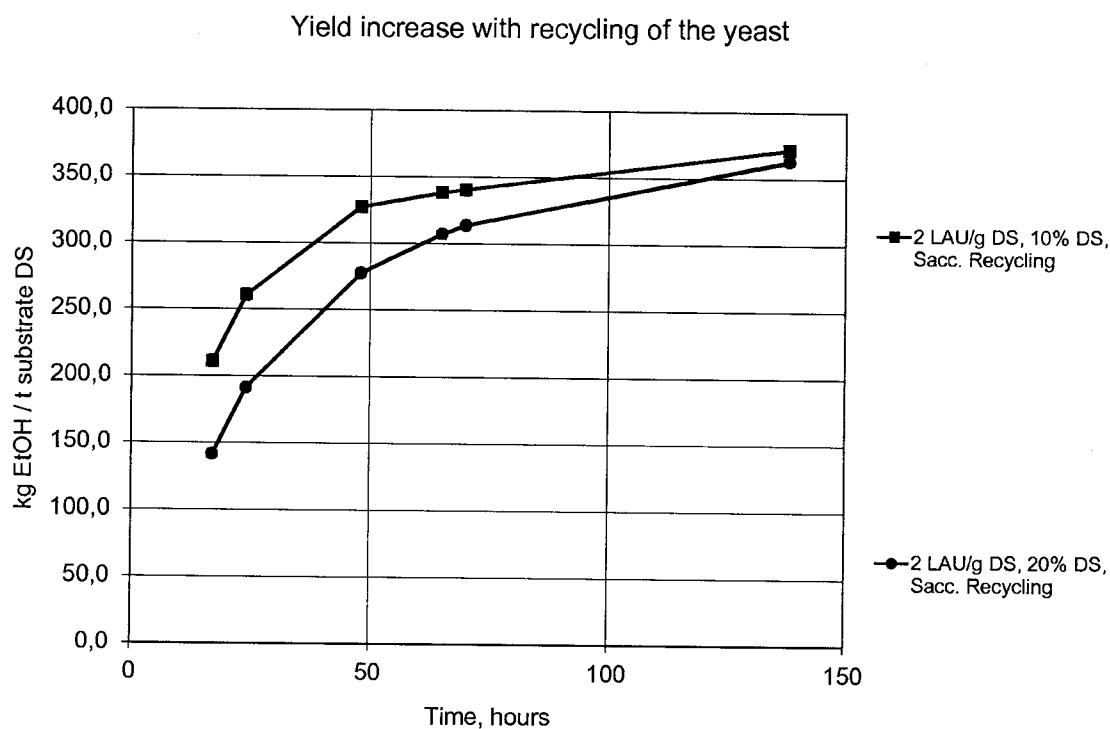
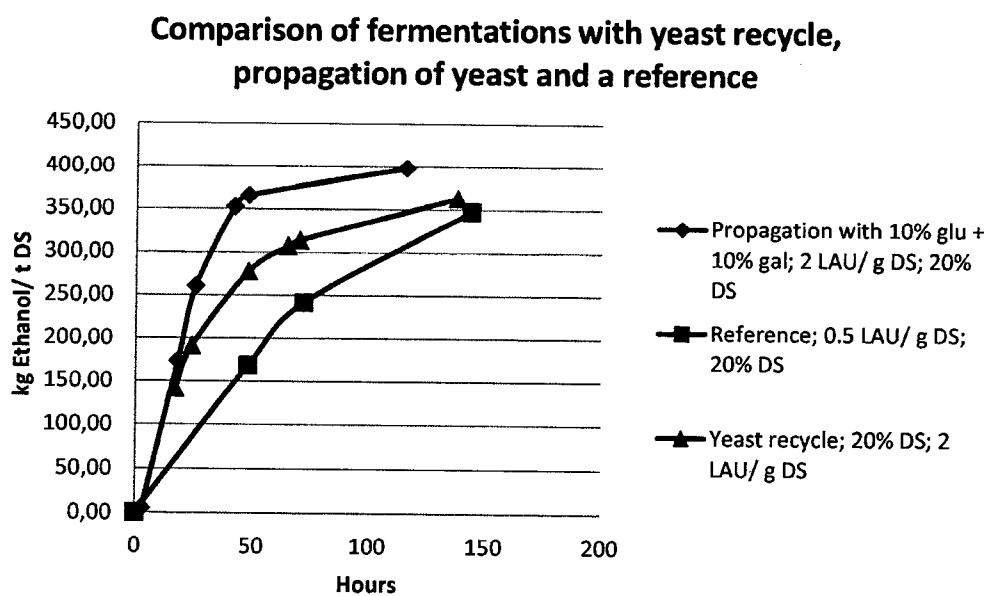


Figure 10.



SSF-fermentation of whey permeate(20% DS)

