Manufacturing of Stabilised Brown Juice for L-lysine production – from University Lab Scale over Pilot Scale to Industrial Production.

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For the last 10 years biotechnological utilization of waste and residues from agriculture and agro-industry has been the common goal for Dansk Biomasse A/S, AgroFerm A/S and Center for Agro-Industrial Biotechnology, University of Southern Denmark.

The concept of the green biorefinery has been described in articles, posters and patent applications and a lot of experimental work in laboratory, pilot and full industrial scale has been carried out in order to implement the ideas for industrial purposes.

Experimental results from laboratory and pilot scale on utilizing brown juice from the green crop drying industry as raw materials in a Danish L-lysine production, will be presented.

On the basis of these results the long and difficult way from idea over lab scale to pilot scale, and last but not least, the latest scale up step from pilot scale to full production scale will be described and discussed.

The evolution from a state university scientific environment to a private company with the aim to set up a profitable large-scale production will be evaluated and discussed as well.

The final result of our work is a fermentation plant using brown juice as growth medium. One single green crop drying factory producing 50 000 tons of fodder pellets a year has enough brown juice to supply a 12 500 tons L-lysine factory with fermentation medium. First step in the production chain is a lactic acid fermentation of the fresh brown juice in the green pellet factory. The lactic acid fermented brown juice is used as fermentation medium in a fermentation process, in which different carbohydrate sources can serve as extra carbon source.

The actual project with scale up from laboratory to pilot scale and finally to large industrial scale production will provide the basis for the illustration of the necessity of realizing the complexity in moving from university laboratory scale to industrial production scale in a private company. We have selected the first step in the process, the pre-treatment of the brown juice to illustrate the process from university laboratory to industrial scale production.

Keywords:
Fermentation, industrial fermentation, continuous fermentation, *Lactic acid*, L-lysine, *Lactobacillus*, the green biorefinery.

Introduction

The green biorefinery

Biorefineries represent a technology for utilization of Renewable Resources and natural compounds in form of green and waste biomass. The term Renewable Resources stands in this case for green crops such as Italian ryegrass, alfalfa, clover, immature cereals from extensive land cultivation, vegetable residues e.g. different kinds of straw and fibres (maize, grain, rape, hemp, flax, etc.), potato and vegetable industry wastes and molasses. The green crops are used in the bio-production of bulk and fine chemicals as well as fodder and energy.

We define "the green biorefinery" as biorefinery where we use renewable raw materials produced in an ecological sustainable way, where the following production process takes into account sustainability, where all the biomass will be used to produce useful products, and no (new) waste materials are produced.

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In the green biorefinery the green crops are fractionated in a press cake containing particles and insoluble high molecular weight compounds and a liquid fraction containing soluble compounds. Vitamins, colours, enzymes and other phytochemicals can be isolated directly from the juice or press cake. The press cake can be used as animal feed or as solid fuel after drying. After extraction of the high value compounds from the juice, it can be used as a substrate for fermentation. The fermentation products can be any organic compounds such as enzymes, antibiotics, biodegradable plastics, organic acids, alcohols, and amino acids.[1]

Brown juice

The green crop drying industry in Denmark uses Italian ryegrass, clover and alfalfa as raw materials for production of green pellets. The green crops are harvested and transported to the green crop-drying factory where it is subjected to a wet separation resulting in a press cake and a juice. In some factories the green biomass is heated to 80 °C by steam before the separation process. This causes the plant cells to burst and the protein to coagulate. The produced waste stream is called brown juice. The typical brown juice dry matter content is 4 to 8 %. Approximately 200 000 m^3 of brown juice are produced each year in Denmark.[2]

The brown juice can be used as fertiliser for green crops, because the juice has a valuable content of nutrients such as potassium and nitrogen. However, there are environmental problems involved in applying excess nitrogen to the green crop fields. If not taken up by plants in the autumn and winter period, excess nitrogen can end up in the ground water as nitrate. Therefore more restrictions have been introduced regarding the use of these residues as fertilizers. In Denmark there is a ban on applying brown juice as a fertilizer from October 1st to February 1st.[2]

Biotechnological utilization of waste and residues from agriculture and agro-industry has been the common goal for the last ten years for Dansk Biomasse A/S, AgroFerm A/S and Centre for Agro-Industrial Biotechnology, University of Southern Denmark. A lot of work has been done on laboratory and pilot scale to examine the potential use of primarily brown juice as a substrate for microbial fermentation, but also the use of other waste products such as potato juice from the potato starch industry has been studied.[3] Using the concept of the green biorefinery a production of lysine feed concentrate on the basis of brown juice from the green crop-drying factory, has been developed.[4]

Lysine fermentation

L-lysine is an essential amino acid used as a fodder additive especially for pigs and poultry. The lysine improves the ability of the animals to digest and utilize nitrogen in the feed, which enhances the growth of the animals and decreases the release of nitrogen into the environment. Lysine is normally produced by fermentation using corn steep liquor (CSL) as growth medium for *Corynebacterium glutamicum* with starch hydrolysate, sugar or molasses as carbon source.

AgroFerm A/S has developed a lysine fermentation process using the acidified brown juice as growth medium.[4]

The acidified brown juice is concentrated to w_{DM} = 25 %, sterilized in a continuous sterilizer and led to a sterile, aerated STR reactor. The initial charge medium is supplemented with necessary minerals, amino acids, vitamins not sufficiently present in the brown juice, sugar, and ammonia. The fermentor is inoculated with a fast growing culture of *Corynebacterium glutamicum* and carried out as fed batch fermentation.

A sterile carbohydrate solution is added continuously to the fermentation tank after the sugar in the initial medium has been utilized. pH of the medium is controlled by addition of ammonia. The fermentation continues until a certain L-lysine mass fraction is achieved. pH in the media is dropped to 4.0 by adding sulphuric acid, and the final liquid product with w = 25 % L-lysine is achieved after vacuum evaporation of the whole media containing all remaining nutrients and biomass. The result is production of a new valuable product with no waste streams.

This article outlines the research that has been done in order to get from idea to production scale. It would be much too comprehensive to do this on the entire process. Therefore, we have selected the first step in the process, the pre-treatment of the brown juice to illustrate the process from university laboratory to industrial scale production. Needless to say that the research done on the part of the process concerning the L-lysine production and the realisation of a 12 500 ton L-lysine factory is much more extensive and complex.

Materials and methods

Materials and methods used to examine the brown juice are all described earlier.[2]

Several strains of *Lactobacillus* were tested in the laboratory e.g.: *L. paracasei* subsp. *paracasei* P 4155, *L. paracasei* subsp. *paracasei* ATCC 25302, *L. paracasei* subsp. *tolerans* DSM 20012, *L. para-*

casei subsp. *tolerans* DSM 20258, *L. delbrueckii*, *L. rhamnosus* ATCC 7469, *L. amylovorus* ATCC 33620, 1001, *L. rhamnosus* ATCC 10863, *L. plantarum* LP1 Christian Hansen, *L. casei* ATCC 11443, *L. helveticus* DSM 20075, *L. salivarius* BC 1001, *L. salivarius* subsp. *salivarius* DSM 20492,[5] *L. salivarius* subsp. *salivarius* DSM 20555.

The abilities of the strains to ferment different carbohydrates were tested by API 50CHL following the manufacturers' instructions (BioMerieux, Marcy-l'Etoile, France).

Fermentations with Lactobacillus strains were done in 2 l fermentors at a temperature of 40 °C, and pH was kept at 7.0 by titration with 4 mol l^{-1} NaOH. The medium was w_{DM} = 5 % brown juice with w = 1.5 % sugar. Propagation of the cells was done in flasks containing w = 5 % brown juice at 40 °C.

Three strains of *L. salivarius* were selected for further testing. The three strains were cultured in 200 ml sterile MRS-broth, inoculated directly from cryo-tube (frozen at –80 °C) without propagation, at different temperatures ranging from 40 °C to 49 °C.

A group of *Bacillus stearothermophilus* kindly supplied by Dr. Herbert Danner, IFA-Tulln, Austria[6] was tested in a w_{DM} = 5 % brown juice medium with w = 3 % sugar: *B. stearotherm*. 125, *B. stearotherm*. 103, *B. stearotherm*. 107 white, *B. stearotherm*. 107 yellow, *B. stearotherm*. 107/01, *B. stearotherm*.113/01, *B. stearotherm*. 117, *B. stearotherm*. 121, *B. stearotherm*. 124. Propagation of the cells was done in shake flasks containing w_{DM} = 5 % brown juice and calcium carbonate added in order to stabilise pH. Fermentations were done in 2 l fermentors at a temperature of 65 °C and pH was kept at 7.2 by titration with 4 mol l^{-1} NaOH.

Continuous fermentation, both, with free cell and with immobilized cell culture was performed in a fluid bed reactor. Temperature was kept at 40 °C by means of a circulating thermo regulated water bath. pH was controlled automatically using NaOH 4 mol l^{-1} as the neutralizing agent. The fermentation medium consisted of brown juice and glucose. The growth medium was w_{DM} = 2 % brown juice with w = 1.5 % sugar. A cryo-tube containing 0.5 ml of a suspension of *Lactobacillus salivarius* was propagated in 40 ml of MRS-broth at 40 °C for 7 to 8 hours before inoculation of the bioreactor.

For entrapment of cells in Ca-alginate gel a sterile w = 5 % sodium alginate solution was prepared. 500 ml of the solution was mixed with 5.46 g cell mass and added drop wise to a sterile w = 5 % calcium chloride solution. Beds of 3 to 4 mm diameter were formed. The beds were inoculated overnight in MRS-broth before filling it in the bioreactor.

Pilot scale experiment with continuous fermentation of brown juice was made in the green crop-drying factory: Dangroent Products, Gredstedbro, Denmark. The brown juice produced from pressing the crops was cooled to approximately 40 °C and let to an 8 m^3 tank. The tank was inoculated with 1 m^3 of a fresh culture of *L. salivarius* grown in sterile brown juice with w_{DM} = 5 %. The flow rate of the brown juice into the fermentor was regulated to keep pH in the fermentor below 4.5.

Results

Brown juice

Initial experiments showed that juice from green biomass could be used as a fermentation medium for micro-organisms demanding vitamins and amino acids.[7]

The next important step was to find a way to effectively preserve the juice. It is well known that green crops and green crop juices decay very rapidly if stored inappropriately. Natural and uncontrolled fermentation processes take place often by a variety of different micro-organisms under uncontrolled conditions. The organic compounds of the juice are then converted to different compounds, often with an unpleasant odour as a result.

Therefore the brown juice has to be subjected to some form of pre-treatment in order to convert it to a stable, storable product that can be used as a complete, universal fermentation medium. Traditional sterilization procedures (sterilization at 121 °C for about 30 minutes) destroy the juice as a fermentation medium due to spoilage of the free amino acids, formation of Maillard-reaction-products and inactivation of enzymes, breaking down the polysaccharides in the juice, making the sugar available for microbial fermentation.

Carrying out an initial lactic acid fermentation is another and much more careful way of effectively preserving the brown juice and at the same time converting it into a suitable fermentation medium. After the separation process in the drying plant, the brown juice has a temperature of approximately 70 °C. In order to run a continuous lactic acid fermentation the hot juice must be cooled to the optimum temperature of the used bacterial strain and led continuously to a stirred tank reactor where an inoculum is added. The lactic acid bacteria convert the sugars in the brown juice to lactic acid, thereby, dropping pH of the juice to about 4.

If the non-concentrated, acidified juice is kept in a cistern at room temperature, it has been shown, that it can be stored for at least 6 months without loss of bio-available amino acids.[4]

The next step in the process was to examine the content of carbohydrates and organic acids in the juice, seasonal variations etc.

Carbohydrate analysis of the brown juice before and after acid hydrolysis showed that the content of free carbohydrates in the juice was increased after hydrolysis (Table 1). This proved that some of the carbohydrate in the juice is bound in long chains of polysaccharides.

Further analysis showed that the content of fructose mainly increases by hydrolysis, indicating that the polysaccharide in the juice consists of fructose: fructans. To utilize these fructants lactic acid bacteria able to hydrolyse the fructans should be used, or sterilization of the brown juice should be avoided to allow the natural enzymes in the juice to do the hydrolysation.[2]

The amount of carbohydrates and organic acids in the brown juice varies according to the composition of the grass (the fractions of Italian ryegrass, clover and alfalfa) and the time of harvest. The highest amount of, both, free sugars and fructan is found in Italian ryegrass about the first week of September. Clover grass has a high content of both free sugars and fructan in the beginning of the season, as alfalfa has a more stable content of free sugars and contains no fructan. (Table 2).

Table 1 – *Chemical composition in w = g kg^{-1} of dry matter in green juice from first cut of different crops, and in one sample of brown juice from Dangroent Products A/S Ringkoebing, Denmark. All samples were taken May /June / July 1998*[2]

Chemical composition of dry matter w/g kg^{-1}	Green juice from alfalfa 10/6–98	Green juice from clover grass 27/5–98	Green juice from Italian ryegrass 2/7–98	Brown juice 9/6–98
Water Soluble Carbohydrates, WSC	137.0 ± 1.3[a]	330.8 ± 1.9	449.4 ± 3.6	462.1 ± 1.3
Free carbohydrates	135.8 ± 1.0	219.5 ± 2.7	283.1 ± 1.9	355.0 ± 8.8
"Fructan"	0	111.3 ± 4.6	166.3 ± 5.5	107.1 ± 10.1
Succinic acid	3.2 ± 0.1	5.7 ± 0.2	15.2 ± 0.4	5.6 ± 0.01
Malonic acid	53.5 ± 0.8	5.7 ± 0.2	17.7 ± 0.2	6.5 ± 0.2
Citric acid	8.3 ± 0.1	14.6 ± 0.2	8.9 ± 0.1	16.3 ± 0.1
Malic acid	33.7 ± 0.8	36.9 ± 0.9	42.8 ± 1.0	24.3 ± 0.01
Acetic acid	0	0	0	5.0 ± 0.1
Lactic acid	0	0	3.3 ± 0.1	63.1 ± 1.0
Formic acid	0	0	4.5 ± 0.2	0
Total organic acids	98.7 ± 1.8	62.9 ± 1.5	92.4 ± 2.0	120.8 ± 1.4
Protein (N x 6.25)	349.0 ± 6.8	264.2 ± 15.3	174.0 ± 1.2	215.0 ± 16.7
Dry matter %	6.02 ± 0.005	5.94 ± 0.01	5.38 ± 0.001	3.38 ± 0.01

[a]Determinations in triplicate

Table 2 – *Fraction variations of free sugars: mono- di-and trisaccharides and fructan in cuts of alfalfa, clover grass and Italian ryegrass. Amounts in w = g kg^{-1} dry matter*[2]

Alfalfa (w/g kg^{-1} matter)				Clover grass				Italian ryegrass			
julian day cut	free sugars	fructan	w_{DM}/%	julian day cut	free sugars	fructan	w_{DM}/%	julian day cut	free sugars	fructan	w_{DM}/%
161	196	0	6.02	147	230	117	5.94	183	264	161	5.38
211	153	0	6.15	182	175	45	4.57	210	249	105	4.62
280	177	0	9.58	218	178	78	5.50	252	198	218	5.07
				259	133	60	3.71	299	232	164	6.59

The amount of free sugars in the brown juice has been found to vary between w_{sugar} = 100 and 360 g kg^{-1} DM and fructans between $w_{fructal}$ = 70 and 170 g kg^{-1} DM (Fig. 1) The lowest total amount of carbohydrate was found in June and a remarkable drop in the content of free sugars was seen at day number 211 and day number 280. As alfalfa is harvested only in a short period around theses days it is likely that the drop is caused by a considerable increase in juice derived from alfalfa in the mixed brown juice.

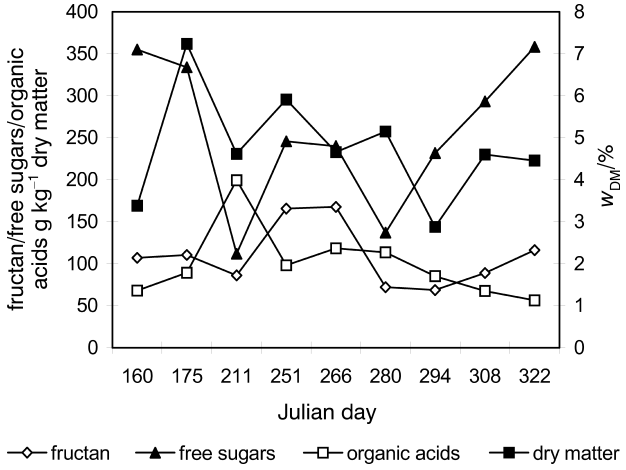

Fig. 1 – *Seasonal variations in content of free and fructan bound sugars, organic acids and dry matter in brown juice*[1]

However, the composition of the brown juice is influenced not only by variations in the amounts of different species of crops but also by weather conditions, temperature, the fall of rain, and the amount of time after harvest where the crops are left in the fields before processing influence the final composition.

Selection of micro-organisms

A comprehensive study was done on the different strains of *Lactobacillus*, with respect to their ability to ferment different carbohydrates, optimum growth rate, and growth in a brown juice medium. Also a group of *Bacillus stearothermophilus* was tested in a brown juice medium.

On the basis of these experiments it was shown that two strains were very effective lactic acid producers: *Lactobacillus paracasei* subsp. *paracasei* P 4155 and *Lactobacillus salivarius* BC 1001. *Lactobacillus paracasei* P 4155 was found to utilize all available free carbohydrates as well as fructans and citric acid, whereas *Lactobacillus salivarius* BC 1001 was not able to utilize fructans and citric acid. In fresh untreated brown juice a very fast conversion of all sugars was found using *L. salivarius* (Table 3). The reason is that enzymes in the untreated juice are converting the fructans to fermentable mono- and disaccharides.

Table 3 – *Yield and lactic acid production of L.salivarius BC1001 and L. paracasei subsp. paracasei P 4155 in sterile and fresh untreated (non-sterile) w_{DM} = 5 % brown juice with w = 1.5% sugar. Experiments were done in duplicate.*

Fermentation in sterile brown juice	Ferm. time *t*/h	Yield $Y_{p/s}$
L. salivarius BC1001	(5)* 21	0.7
L. paracasei P 4155	(25)* 42	0.8
Fermentation in fresh untreated brown juice	**Ferm. time *t*/h**	**Yield $Y_{p/s}$**
L. salivarius BC1001	(4,0)* 18	0.9
L. paracasei P 4155	(10.5)* 18	1.0

* Time of exponential lactic acid production (on the basis of NaOH titration)

Also a group of *Bacillus stearothermophilus* was tested. Four of the strains showed good growth and lactic acid yield (Table 4).

Table 4 – *Yield and lactic acid production of four strains of Bacillus stearothermophilus in w_{DM} = 5 % brown juice with w = 3 % sugar. Experiments were done in duplicate.*

Micro-organism	Ferm. time *t*/h	Yield $Y_{p/s}$
B. stearotherm. 125	8	0.9
B. stearotherm. 107/01	6	0.8
B. stearotherm. 117	9	1.0
B. stearotherm. 124	12,5	1.0

The most effective and cheap way to produce acidified brown juice will be a continuous fermentation in fresh untreated brown juice, therefore it is important to choose a robust fast growing strain. In that respect *L. salivarius BC 1001* is the most promising of these strains, as it grows very fast. Therefore this strain was selected for further lab and pilot-scale experiments.[2]

Continuous fermentation

Continuous fermentation experiments were carried out in the laboratory with, both, free cells of *L. salivarius* and Ca-alginate immobilised cells. The main purpose of these experiments was to examine the robustness of *L. salivarius* BC 1001 in fresh untreated brown juice (running continuously through the bioreactor) for a long period of time.

Free cell continuous fermentations were performed at pH 6.0. It was found that running the fermentation at this pH provoked contamination of the non-sterile substrate, thereby causing the sugar in the medium to be utilized without production of lactic acid. Due to this problem it was decided to run subsequent fermentations at pH 5.5. It was found by a series of batch fermentations at different pH that pH could be lowered to 5.5 without reducing the performance of the bioreactor significantly.

At the beginning of the fermentation, flocculation of the cells and growth on the inner surface of the bioreactor, were observed. This gave us the reason to believe that cells of *L. salivarius* might immobilize themselves during an industrial process. Flocculation of the cells was confirmed by phase contrast microscopy, (Fig. 2). This immobilization was unfortunately very sensitive to the addition of the 4 mol l^{-1} base, and therefore it was decided to immobilise the cells by entrapment in Ca-alginate gel to examine how the process would proceed with an immobilized culture.

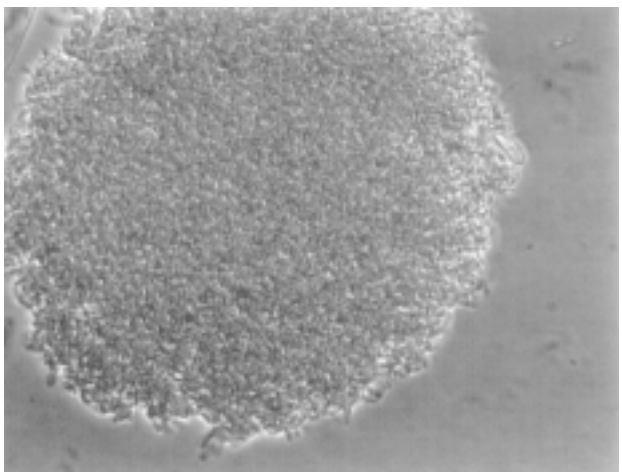

Fig. 2 – *Phase contrast microscopy of flocculating cells of L. salivarius (and presumably other micro-organisms in the non-sterile substrate) taken at day 4 during continuous fermentation of brown juice*

Continuous fermentations with entrapped cells showed lactic acid production of 12.3 g l^{-1} h^{-1} and no decline in productivity at dilution rates over the maximum growth rate (Fig. 3).

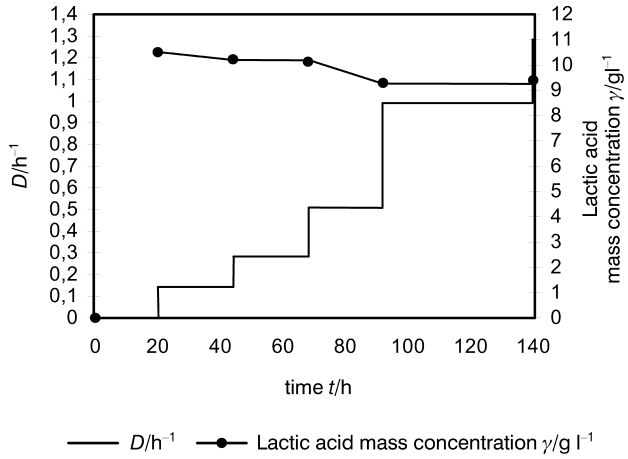

Fig. 3 – *Lactic acid concentration as a function of dilution rate during continuous fermentation with Ca-alginate immobilised cells*[3]

Pilot scale experiments

The aim of the pilot scale experiment was to examine if the continuous fermentation experiments in the laboratory could be succeed also in industrial scale, and to find out whether the strain of *L. salivarius* was robust enough for industrial use. In this continuous fermentation parameters such as temperature and pH could not be kept as constant as in the laboratory runs. Also the amount of carbohydrates in the juice fluctuated depending on the type of crop harvested.

pH in the fermentation tank was fairly stable around 4.5, even though the process parameters fluctuated (Fig. 4).

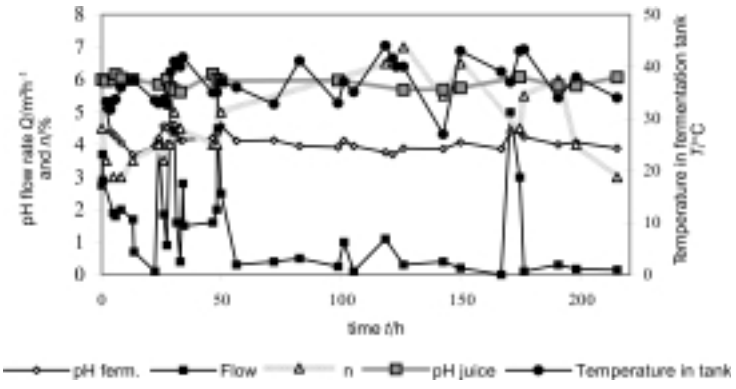

Fig. 4 – *Flow rate of brown juice, dry matter content expressed as refractive index n, pH of the brown juice and pH in the fermentation tank in an 8 m*[3] *continuous fermentation*[3]

The concentration of lactic acid in the fermentation tank was high while concentrations of acetic acid and succinic acid were low (Fig. 5).

Even though the process parameters fluctuated, good lactic acid production was achieved and it

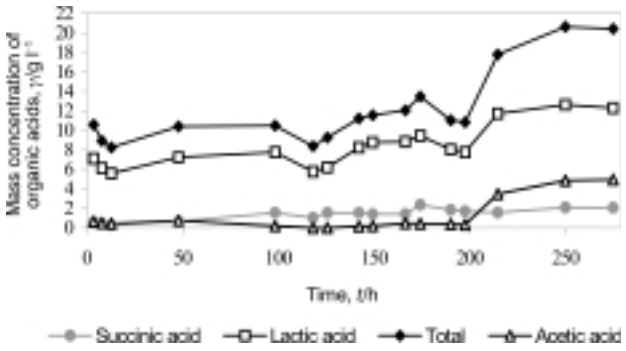

Fig. 5 – *Mass concentration of organic acids in the fermentation tank in an 8 m³ continuous fermentation*

seemed that *L. salivarius* in co-operation with other bacteria in the non-sterile substrate was able to keep pH in the fermentation tank between 4 and 4.5 even though the optimum growth conditions could not be achieved.

Development of an industrially robust process

Pilot scale experiments showed us that process parameters can be very unstable and fluctuations in temperature, flow rate and pH of the brown juice are very likely to occur. The strain used for the pilot scale experiment was *Lactobacillus salivarius* BC 1001, but still we had not decided on this micro-organism as the one to use industrially.

A number of criteria were set up for selecting the lactic acid bacteria to be used industrially (Table 5).

For an industrial process, strains of *Lactobacillus* are preferred over strains of *Bacillus stearothermophilus* because of the status of *Lactobacillus* as GRAS*, and because a lot of the strains are already used in the dairy and food industry, which will ease the procedure of getting the acidified brown juice approved for production of animal feed. Furthermore, the strains of *Bacillus stearothermophilus* showed long lag-phases in fermentation experiments and were not as easy to handle and propagate as the strains of *Lactobacillus.*

Three strains of *L. salivarius* were selected as the best strains to meet the criteria (Table 5). These strains are very fast growing, have a very short lag-phase (even at the first transfer from frozen cryo-tube to medium), keep a high growth rate even at temperatures over 40 °C, and acidify the medium rapidly to pH 4 to 4.5. *L. salivarius* is homofermentative and produces primarily L-lactic acid. Also it seems that cells of this strains has a tendency to flocculate (Fig. 2).

Maximum growth rates of more than 2.0 were observed for *L. salivarius* subsp. *salivarius* DSM

*Generally recognized as safe

Table 5 – *Criteria for selecting a micro-organism to be used for acidification of brown juice*

Robust/fast growing strain	The industrial acidification process should preferably be run at non-sterile conditions to keep the process simple and to avoid expenses for sterilisation of process equipment and of the brown juice, therefore the micro-organism must be fast growing in order to compete with other micro-organisms in the brown juice. Furthermore, the strain should be easy to propagate and handle, because this will be done by personnel in the green crop-drying factory (not educated in microbiology).
Homofermentative strain	Lactic acid bacteria ferment sugars by different metabolic pathways, some strains produce exclusively lactic acid (homofermentative strains), and other strains produce lactic acid, carbon dioxide, ethanol or acetic acid (heterofermentative strains). The micro-organism should preferably be homofermentative, because production of gas can complicate the process, especially if the micro-organisms are immobilized in the bioreactor.
Acidiophilic	pH of the brown juice should be lowered to 4 to 4.5 by the acidification process in order to obtain long durability of the brown juice.
Termofilic	The brown juice has a temperature of 70 °C after the separation process. The micro-organism should be able to grow at at least 40°C in order to keep the amount of water for cooling at a reasonably level. A high fermentation temperature will also reduce the risk of contamination with other micro-organisms.
Approved for food and feed products	Procedures for approval of micro-organisms for food and feed production are expensive and time-consuming, it would therefore be advantageous if the micro-organism is already approved for this purpose.
Facultative anaerobe	Most lactic acid bacteria are facultative anaerobes, but a few show poor growth in the present of oxygen (obligate anaerobes). In a simple industrial process like this, it can be difficult to keep the fermentor completely free of oxygen, and therefore the micro-organism should be facultative anaerobic.
Ability to flocculate	Immobilization of the micro-organism by naturally flocculation would be advantageous, because a higher production rate can be achieved with immobilized cells, and immobilisation techniques such as entrapment in Ca-alginate gels is not robust enough for the industrial process.

20492 and *L. salivarius* subsp. *salivarius* DSM 20555 at 46 °C, but the growth rate was not constant probably due to inhibition of the growth as a result of lactic acid production (drop in pH). Average growth rates were close to 1.0 for *L. salivarius* subsp. *salivarius* DSM 20492 and *L. salivarius* subsp. *salivarius* DSM 20555 at temperatures from 45 to 48 °C, and at 45 °C for *L. salivarius* BC 1001. The highest amount of L-lactic acid was produced at 46 °C for *L. salivarius* subsp. *salivarius* DSM 20492 and *L. salivarius* subsp. *salivarius* DSM 20555 and at 40 °C for *L. salivarius* BC 1001 (Table 6).

The experiments show that either *L. salivarius* subsp. *salivarius* DSM 20492 or *L. salivarius* subsp. *salivarius* DSM 20555 will be the best strain to use in the acidification process, because they have a higher temperature optimum than *L. salivarius* BC 1001. We need to test the three strains further in continuous fermentations at pH 4.5, to see which one of them will be most robust. Also we must test if flocculation of cells can be achieved at this pH.

From idea to reality

On the basis of chemical analyses of the juice, laboratory as well as large pilot scale fermentation experiments and storage stability tests, we are now designing a plant for production of 10 000 tons of acidified, concentrated (w_{DM} = 25 %) brown juice a year.

In order to realize our ideas we have been forced to combine all our experience from the university environment with the practical experience from our pilot scale experiments at a running drying plant

From laboratory experiments in the university as well as literature we have identified:

– Fast growing strains of lactic acid bacteria with a high optimum temperature for growth.

We have also gained experience and know-how about the influence of different factors on the microbial fermentation process etc.:

– Influence of temperature on growth rate and lactic acid production.

– Stability of the fresh and fermented juice under aerobic and anaerobic conditions

– Possibilities of preventing surface growth in storage tanks.

– Sedimentation characteristics of precipitated proteins

From the industrial side we have learned that:

– Brown juice is only available in the growing season of grass, from May 15th to November 15th.

Table 6 – *Growth and lactic acid production of three strains of L. salivarius at temperatures ranging from 40 °C to 49 °C.*

μ_{max}	*L. salivarius* BC 1001	*L. salivarius* subsp. *salivarius* DSM 20492	*L. salivarius* subsp. *salivarius* DSM 20555	pH	*L. salivarius* BC 1001	*L. salivarius* subsp. *salivarius* DSM 20492	*L. salivarius* subsp. *salivarius* DSM 20555
40 °C	0.9	0.87	0.88/0.98	40 °C (8 h)	4.4	4.4	4.6
45 °C	1.4	1.6	1.8/1.4	45 °C (8 h)	4.5	4.3	4.7
46 °C	1.71	2.18	2.2	46 °C (8 h)	4.6	4.4	4.7
47 °C	1.27	1.37	1.84	47 °C (8 h)	4.9	4.6	4.6
48 °C	0.63	1.45	1.47	48 °C (7 h)	5.55	4.93	4.86
49 °C	0.28	0.62	0.641	49 °C (10 h)	5.9	5.2	5.1
$\mu_{average}$	*L. salivarius* BC 1001	*L. salivarius* subsp. *salivarius* DSM 20492	*L. salivarius* subsp. *salivarius* DSM 20555	L-lactic acid γ/g l^{-1}	*L. salivarius* BC 1001	*L. salivarius* subsp. *salivarius* DSM 20492	*L. salivarius* subsp. *salivarius* DSM 20555
40 °C	0.54	0.58	0.34	40 °C (8 h)	13.62	13.84	9.05
45 °C	0.93	1.04	1.08	45 °C (8 h)	9.33	9.54	7.51
46 °C	0.82	0.93	0.98	46 °C (8 h)	8.90	14.58	10.29
47 °C	0.69	0.95	0.90	47 °C (8 h)	4.63	8.46	8.37
48 °C	–	1.44	1.02	48 °C (7 h)	3.43	7.60	8.06
49 °C	–	–	0.453	49 °C (10 h)	1.14	3.64	4.17

– The lysine factory uses about 200 tons of concentrated brown juice every week all year round.

– Availability of brown juice is very much dependent on weather conditions varying from 0 during dry summers to 90 $m^3\ h^{-1}$ in the wet autumn.

– The quality of the pressed juice can vary a lot and as a result quality control is necessary.

– At least half of the juice must be returned to the drying factory and added to the press cake in order to make good fodder pellets.

– The evaporator is using surplus heat from the dryer and is only running during the green crop season.

On the basis of these conditions we have set up a flow diagram for the industrial acidification process (Fig. 6).

The hot brown juice from a green crop drying plant is at the first step cooled to fermentation temperature.

We have decided to use only the best quality of the juice in the acidification process and return the other half of the juice to the pellet factory in concentrated form. In order to select a high quality brown juice for the lactic acid fermentation we split up the juice stream in two separate streams on the basis of pH measuring (perhaps also other quality criteria will be taken into account).

If pH is more than 5.5; the brown juice is led to lactic acid fermentation in a 100 m^3 CSTR inoculated with *Lactobacillus salivarius.* If pH is below 5.5; the brown juice is led to the sediment buffer tank, where also the sediment from the sedimentation tank is led. After evaporation, the concentrated brown juice is used in the production of fodder pellets in the Drying Plant.

The acidified brown juice with pH between 4.0 and 4.5 is led to a sedimentation tank, from where the supernatant is led to a storage/buffer tank before evap-

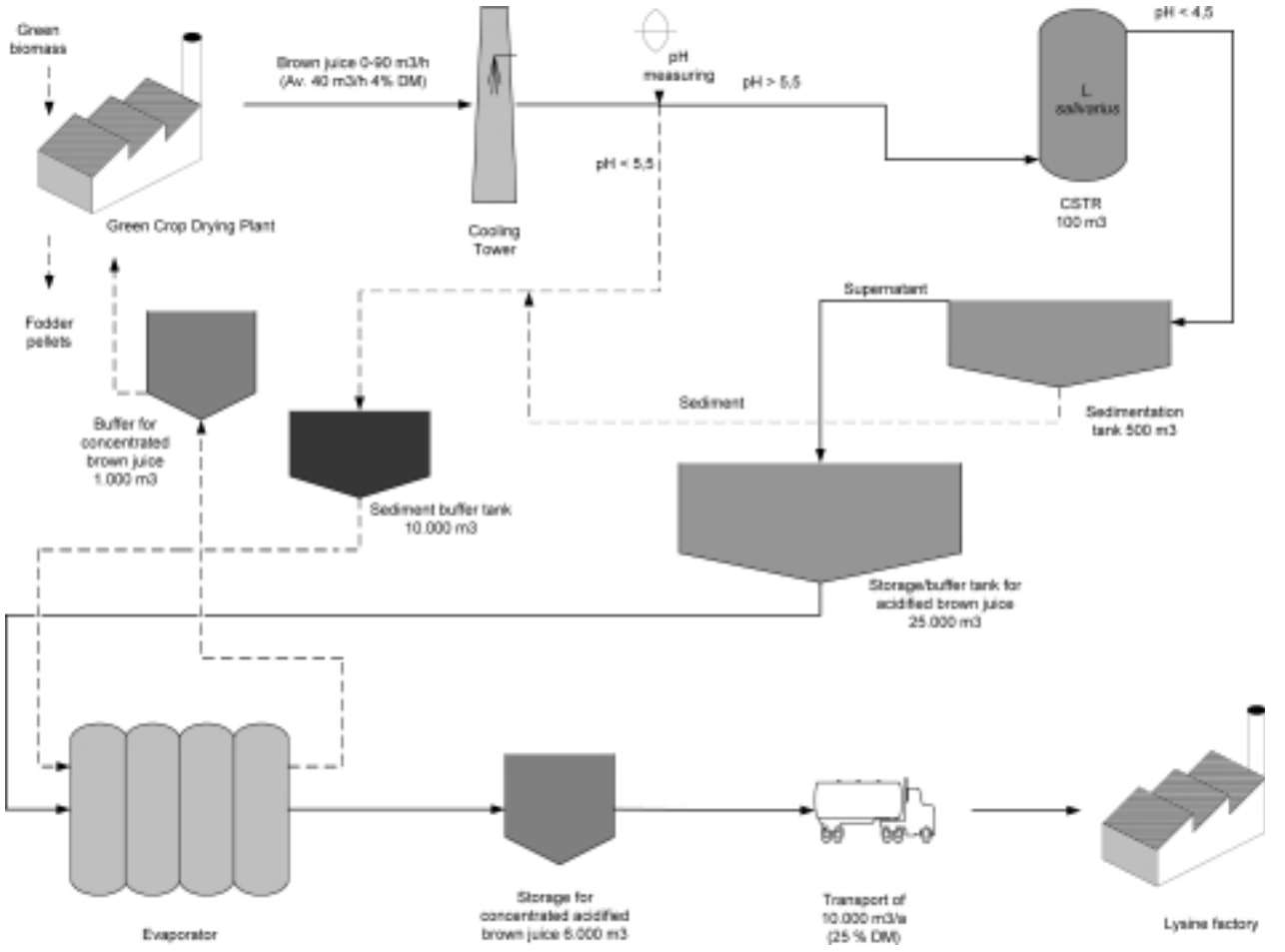

Fig. 6 – *Brown juice from a Green Crop Drying Plant is cooled to fermentation temperature and on the basis of pH measuring split up into two directions: 1. pH > 5.5: the brown juice is led to lactic acid fermentation in a 100 m^3 CSTR inoculated with Lactobacillus salivarius. The acidified brown juice (pH < 4.5) is led to a sedimentation tank, from where supernatant is led to a storage/buffer tank before evaporation to w_{DM} = 25 %. The sediment is led to a sediment buffer tank. The concentrated acidified brown juice is stored until transport to the lysine factory. 2. pH < 5.5: the brown juice is led to the sediment buffer tank, where also the sediment from the sedimentation tank is led. After evaporation, the concentrated brown juice is used in the production of fodder pellets in the Drying Plant.*

oration to w_{DM} = 25 %. The sediment is led to a sediment buffer tank. The concentrated acidified brown juice is stored until transport to the lysine factory.

Using this concept it is possible to supply the lysine factory with stabilized high quality brown juice all year round and produce about 50 000 tons of liquid lysine feed concentrate a year.

Symbols/abbreviations

w_{DM} – mass fraction of dry matter, %
D – dilution rate, h^{-1}
n – refractive index, %
μ – specific growth rate, h^{-1}
w – mass fraction, %
DM – dry matter
γ – mass concentration, g l^{-1}
t – time, h

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