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High density process to cultivate *Lactobacillus plantarum* biomass using wheat stillage and sugar beet molasses

Małgorzata Krzywonos[1] ✉ · Thomas Eberhard[2]

1 Department of Bioprocess Engineering, Wrocław University of Economics, Poland

2 Department of Microbiology, Uppsala Genetic Centre, Swedish University of Agricultural Sciences, Sweden

✉ Corresponding author: malgorzata.krzywonos@ue.wroc.pl
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ABSTRACT

Background: Owing to the growing interest in biofuels, the concept of a biorefinery where biomass is converted to a variety of useful products is gaining ground. We here present how distillery waste is combined with a by-product from a sugar production, molasses, to form a medium for the growth of *Lactobacillus plantarum* with yields and biomass densities comparable with conventional industrial media. Such approach enables a cost-effective utilization of the problematic wastewater from ethanol and a by-product from sugar production. It is the first approach that attempts to find low-cost media for the production of *Lactobacillus plantarum* biomass.
Results: This study suggests that sieved wheat stillage enriched by adding 1.77 g/l yeast extract and 10% molasses (v/v), with NH_4OH used for pH adjustment, may be used as a media for large-scale cultivation of *L. plantarum.* Such composition of the medium permits a high density of lactic acid bacteria (1.6×10^{10} cfu/ml) to be achieved.
Conclusions: The use of a fermentation medium consisting of distillery wastewater and molasses to obtain value-added products (such as LAB biomass and lactic acid) is a possible step for classical ethanol production to move towards a biorefinery model production in which all by and waste products are utilized to increase produced values and reduce waste production. This enables a cost-effective utilization of the problematic wastewater from ethanol and sugar production.

Keywords: distillery wastewater, high density fermentation, lactic acid bacteria, *Lactobacillus plantarum,* sugar beet molasses, wheat stillage

INTRODUCTION

Lactic acid bacteria (LAB) have been used by humans since prehistoric times in the production of various foods as preservatives and to add flavour (Briens et al. 2008). The Nobel laureate Ilya Mechnikov introduced the concept of using bacteria in food to modify the gut microflora to improve human health, calling the concept probiotics. Recently probiotics have also been used in animals. European restrictions on the use of antibiotics in feed for farm animals have enhanced interest in alternative to antibiotics to curb infectious diseases at farms. One such alternative is the large-scale application of probiotic bacteria in animal feed (Gaggia et al. 2010); this is why the potential market for LAB biomass is greatly increasing (Schiraldi et al. 2003; Briens et al. 2008; Wohlgemuth et al. 2010).

LAB are reported to be extremely fastidious organisms with numerous growth requirements. They need rich media containing compounds such as amino acids, peptides, vitamins and nucleic acids (Narayanan et al. 2004; Dumbrepatil et al. 2008). MRS (De Man, Rogosa, Sharpe broth) is amongst the media most commonly used for *Lactobacillus* growth; nevertheless, it has some major

disadvantages, *e.g*. a complex composition. Another major disadvantage is that the medium includes components too costly for industrial applications.

One of the most widespread *Lactobacillus* species used in food technology is *Lactobacillus plantarum.* Pertinent studies reported in the literature have been conducted to find less expensive media supporting *Lactobacillus* growth. Those found contained carbohydrate and/or nitrogen sources such as wheat flour hydrolysate (Hofvendahl and Hahn-Hägerdal, 1997), wheat bran (Naveena et al. 2005), hydrolysates of wheat bran combined with corn steep liquor (Li et al. 2010), lactose and whey permeate (Fu and Mathews, 1999), whey solely (Mondragón-Parada et al. 2006), hydrolysates of fish viscera (Horn et al. 2005), corn steep liquor, cane molasses with animal and marine by-products (Demirci et al. 1998), and molasses solely (Tondee and Sirianuntapiboon, 2008). It is essential to note that all of those studies were aimed at optimizing the medium for lactic acid production. Only a few reports can be found in the literature on a high density processes to culture lactic acid biomass *e.g. Lactobacillus casei* (Aguirre-Ezkauriatza et al. 2010) and *Lactobacillus delbrueckii* ssp. bulgaricus (Schiraldi et al. 2003). The work reported on in this paper is the first one that attempts to find low-cost media for the production of *Lactobacillus plantarum* biomass.

LAB are typically used as starter cultures in the dairy industry. They may also be used as preservatives in fodder (Briens et al. 2008; Ye et al. 2008). In such applications, they are known to inhibit the growth of microbial pathogens and spoilage organisms, or increase digestibility; but they may also display probiotic effects. Another use for LAB is as an animal probiotic in its own right (Gaggia et al. 2010). However, the use of LAB are not restricted to food and feed, they have recently been used in weed control on golf courses (Omer et al. 2010).

The aim of this study was to obtain a low-cost medium for an industrial scale production of *Lactobacillus plantarum* biomass*.* For this purpose, a preliminary experimental study was conducted, where we used wheat stillage and sugar beet molasses as carbohydrate and/or nitrogen sources.

Table 1. Characterization of wheat stillage.

Parameter	Content
pH	4.0–4.1
Water content [%]	95–97.1
$N\text{-}NH_4$ [g/kg]	0.5–0.8
Protein nitrogen x 6.25 (Kjeldahl) [%]	1.2–2.3
Phosphorus, P [mg/l]	54–60
Glucose [g/ 100 g]	< 0.04
Lactic acid [g/l]	3.26
Acetic acid [g/l]	5.52
Propionic acid [g/l]	7.28
Butyric acid [g/l]	0.65

METHODS

Microorganism

Lactobacillus plantarum MiLAB 393 was kept frozen in a 20% (w/v) glycerol solution until use.

Inoculum preparation

The preparation of inoculum commenced with the transfer of the frozen microorganism to a 100 ml flask containing 50 ml of MRS medium (Oxoid CM0359B, Basingstoke, England) under aseptic conditions. The flask was incubated at 37ºC for 24 hrs before use in the experiments. Inoculum volume for biomass production comprised 7.5 ml of bacteria grown on MRS medium.

Media

Distillery wastewater (wheat stillage, by-product of ethanol distillation, with parameters itemized in Table 1) was used as a medium for bacteria cultivation. Before use, solid particles were removed from the stillage in two ways: (1) filtration through filter paper, or (2) sieving (0.45 mm mesh). The liquid phases obtained after separation were stored at 4ºC before incorporation into growth media.

Wheat stillage was enriched with the following ingredients: glucose (G; BHD, VWR International, Leuven, Belgium; 20 g/l), molasses (M; Danisco, Malmö, Sweden; 5, 10% v/v), salts (S; 5 g/l K_2HPO_4, Scharlau PO 0258, Barcelona, Spain; and 0.75 g/l $MgSO_4$, Scharlau MA 0085, Barcelona, Spain), peptone (P; Oxoid LP LP0037B, Basingstoke, England; 5.08 g/l), and yeast extract (YE; Oxoid LP 0021, Basingstoke, England; 1.77, 2.65, 3.54, 5.31, 7.08 and 10.62 g/l). Glucose and molasses were added separately after sterilization of the stillage and additives at 121ºC for 15 min.

Process conditions

All fermentations were conducted at 37ºC in 250 ml shake flasks (containing 150 ml of the medium) at 120 rev/min (Lab-Term incubator, Adolp Kühnes AG, Schweiz). The pH was held at 6.0 by the automated addition of 25% (w/v) NaOH or 25% NH_4OH (Scharlau, Sentemenat, Spain). The pH was controlled by a pH control unit (Inventron, Kungsbacka Sweden) using P2 peristaltic pumps (Belach, Solna, Sweden). The autoclavable electrodes (Bradly James, Irvine California, USA) were used to measure pH value. The analogue output from the pH control unit was sent to an NI-USB-6218 AD converter, which is connected to a computer. National Instruments VI logger software has been used to collect the data.

All experiments were conducted aseptically in duplicate and average values are reported.

Table 2. Supplementation of filtered stillage.

Composition of the medium*	Glucose conversion [%]	Max LA [g/l]	Maximal number of LAB [cfu/ml]	$Y_{P/S}$ [g/g]**	Q_P [g/(l·h)]**
Stillage	-	0.98	4.8×10^5	-	-
Stillage + Glu	12.7 [48 h]	2.9 [24 h]	4.4×10^7	0.89	0.08
Stillage + Glu + Salts	78.9 [36 h]	14.8 [36 h]	9×10^7	0.99	0.62
Stillage + Glu + Salts + P	100 [24 h]	19.5 [24 h]	4×10^9	1.11	0.81
Stillage + Glu + Salts + YE	100 [12 h]	19.6 [24 h]	4.4×10^9	1.04	0.82
Stillage + Glu + Salts + YE + P	100 [12 h]	20.5 [12 h]	31.8×10^9	1.06	0.85
Stillage + M	63.6 [48 h]	15.5 [48 h]	8.9×10^8	0.84	0.64

*****YE = Yeast Extract (5.31 g/l), Glu = Glucose (2%), M = Molasses (5%), Salts (5 g/l K_2HPO_4; 0.75 g/l $MgSO_4$), P = Peptone (5.08 g/l). **Glucose conversion, $Y_{P/S}$ and Q_P were calculated for values obtained in the 24 hrs of the process.

Analytical methods

Increases in biomass (cfu/ml) were determined by plating serial dilutions on MRS agar. The plates were assessed after 48 hrs of incubation at 37ºC. The concentrations of organic acids (lactic, propionic, acetic, isobutyric and butyric) and sugars (glucose, fructose and sucrose) were analyzed by HPLC (column, Rezex ROA Organic Acid H^+ (8%), 300 x 7.8 mm, Phenomenex, at ambient temperature; eluent: 5 mM H_2SO_4 at 0.6 ml/min; injection, 5 µl; detector, RID).

Sugars content was expressed as glucose concentration to obtain comparable results. Glucose conversion was calculated as the difference between the initial and final concentration in the medium in proportion to the initial concentration value. The maximum concentration of lactic acid produced (max LA, g/l) was calculated as the maximal detected value minus the initial lactic acid content. Lactic acid

yield ($Y_{P/S,}$ g/g) was expressed as the lactic acid produced in proportion to glucose consumed by the bacteria on a weight basis (g/g). Calculations were performed for glucose equivalent in order to obtain comparable results. Volumetric productivity of lactic acid (Q_P, (g/(l·h))) was defined as the maximum quantity of lactic acid formed per litre of fermentate per hr.

RESULTS

Supplementation of filtered stillage

Table 2 summarizes the results obtained when wheat stillage filtrate (filter paper) was used as the fermentation medium for LAB growth, with and without combinations of supplements. With non-supplemented stillage, the maximal quantity of lactic acid produced (max LA) and the maximal number of LAB were only 0.98 g/l and 4.8 x 10^5 cfu/ml, respectively. The composition of the wheat stillage (Table 1) suggests that the low level of sugars may have been a limiting factor in the process. The addition of glucose (2%) had a beneficial effect on both maximal number of LAB and max LA, which totaled 4.4 x 10^7 cfu/ml and 2.9 g/l, respectively. Addition of glucose solely increased the max LA value approx. threefold (Table 2). However, only 12.7% of the glucose added was converted during fermentation (48 hrs). This might be the reason why the theoretical yield of LA content was as low as 16%. The addition of salts (K_2HPO_4, $MgSO_4$) enhanced glucose conversion 6.2-fold (to 78.9%) and max LA concentration 5.2-fold (to 14.8 g/l). There was also a 7.4-fold increase in productivity and a 9.8% increase in lactic acid yield. Substantially higher values were obtained using a medium enriched with glucose, salts and peptone. In that medium, the maximal number of LAB increased to 4 x 10^9 cfu/ml, and the max LA produced was by 24% higher than in the similar medium with no peptone enrichment. All the glucose present in the medium was utilized during the first 24 hrs of the process.

Replacement of peptone with yeast extract (YE) produced similar results. The maximal LAB grown and LA produced were 4.4 x 10^9 cfu/ml and 19.6 g/l, respectively. Supplementation of the stillage with glucose, salts, and both peptone and YE raised the maximal number of LAB and LA concentration to 31.8 x 10^9 cfu/ml and 20.5 g/l, respectively (Table 2). In order to improve the cost-effectiveness of the process, glucose was replaced with sugar beet molasses (5%). In the molasses-enriched medium the assimilation of carbohydrates (expressed as glucose equivalent), the maximal number of LAB, and the max LA synthesized were higher (63.6%, 8.9 x 10^8 cfu/ml, 15.5 g/l) whereas lactic acid yield remained at approximately the same level (0.84 g/g). The value of the theoretical lactic acid yield for the molasses-enriched medium was 3-fold higher than the value obtained for the pure-glucose supplemented medium. The highest LA productivity (Q_P) (0.85 g/(l·h)) was observed when the medium was enriched with glucose, salts, YE and peptone. The lowest Q_P value was obtained in the experiment where glucose was the sole supplement (0.08 g/(l·h)) (Table 2).

Table 3. Supplementation of filtered stillage with yeast extract (YE) and molasses (5% and 10%); pH controlled with NH_4OH.

Parameter	5%M			10%M		
	2.65g/l YE	5.31g/l YE	10.62g/l YE	2.65g/l YE	5.31g/l YE	10.62g/l YE
Glucose conversion [%]*	100	100	100	67.2	99.9	99.8
Max LA [g/l]	21.6 [32 h]	25.0 [32 h]	27.6 [24 h]	38.6 [48 h]	41.2 [24 h]	40.9 [24 h]
Maximal number of LAB [cfu/ml]	2.4 x 10^9 [12 h]	2.8 x 10^9 [12 h]	3.4 x 10^9 [12 h]	3.1 x 10^9 [12 h]	3.0 x 10^9 [12 h]	3.6 x 10^9 [12 h]
$Y_{P/S}$ [g/g]*	0.74 (74%)[a]	0.84 (84%)[a]	0.945 (94.4%)[a]	0.665 (44.7%)[a]	0.895 (89%)[a]	0.86 (86%)[a]
Q_P [g/(l·h)]*	0.89	1.04	1.15	0.94	1.72	1.70

*Glucose conversion, $Y_{P/S}$ and Q_P were calculated for values obtained in the 24 hrs of the process. [a]% of the theoretical yield.

Filtered stillage supplemented with yeast extract and molasses

In this experimental series (Table 3), a medium enriched with molasses (5% and 10%) was supplemented with various amounts of YE; the neutralizing agent being NH_4OH. The maximal number of LAB produced ranged from 2.4 to 3.62 x 10^9 cfu/ml in all formulations. Doubling the quantity of molasses and increasing the quantity of YE 4-fold caused the LAB biomass and LA production to rise (Table 2 and Table 3).

In the processes using molasses-enriched media (5% addition), the yield of lactic acid production ($Y_{P/S}$) increased with the amount of YE added, the highest value being measured at the highest YE concentration (10.62 g/l) (Table 3). With 10% molasses, the highest values of $Y_{P/S}$ and Q_P were observed at a considerably lower YE concentration (5.31 g/l) (Table 3).

Supplementation of sieved stillage

These experiments were conducted in the liquid phase of the stillage after removal of solids by sieving (0.45 mm). The aim was to examine how the method of solids separation affects LA synthesis and LAB growth. The results are summarized in Table 4. When the sieved stillage was not enriched, the max LA value (1.08 g/l) and the maximal number of LAB (5.7 x 10^5 cfu/ml) were similar to those achieved with the non-enriched filtered stillage (Table 2). When sieved stillage was supplemented with glucose, the maximal number of LAB (4.6 x 10^8 cfu/ml) was higher than that supported by either the non-supplemented sieved stillage (Table 4) or the filtered glucose stillage (Table 2). The replacement of glucose with molasses increased the max LA value and the maximal number of LAB (17.11 g/l and 9.5 x 10^8 cfu/ml, respectively). The values of these parameters, as well as those of $Y_{P/S}$ and Q_P, were higher than the values attained using filtered stillage with either glucose or molasses enrichment (Table 2 and Table 4).

Table 4. Supplementation of sieved stillage.

Composition of the medium	Glucose conversion [%]	Max LA [g/l]	Maximal number of LAB [cfu/ml]	$Y_{P/S}$ [g/g]*	Q_P [g/(l·h)]*
Stillage	-	1.08	5.7 x 10^5 [24 h]	-	-
Stillage + 2%Glu	14.4 [48 h]	3.64 [48 h]	4.6 x 10^8 [48 h]	1.34 (19%)[a]	0.15
Stillage + 5% M	70.7 [48 h]	17.11 [48 h]	9.5 x 10^8 [48 h]	0.87 (62%)[a]	0.71

*Glucose conversion, YP/S and QP were calculated for values obtained in the 24 hrs of the process. [a]% of the theoretical yield.

Sieved stillage with molasses and yeast extract supplementation

Control of pH with NaOH. The aim of the experiments was to study how the addition of YE (2.65, 5.31 or 10.62 g/l) and molasses (5% or 10%) influences the values of LAB growth, LA production, $Y_{P/S}$ and Q_P, when sieved stillage was used as a medium, and the pH was controlled using NaOH. Relevant data are compiled in Table 5. The maximal number of LAB ranged between 1.2 x 10^9 and 4.8 x 10^9 cfu/ml. Addition of 5% molasses and 2.65 g/l YE produced the lowest number of LAB, whereas that of 10% molasses and 10.62 g/l YE accounted for the highest growth of LAB. LAB numbers peaked at 12 and 24 hrs of the process. The data in Table 5 demonstrate that varying YE concentrations in the medium failed to influence the amount of LA produced, and that the addition of molasses enhanced LA synthesis. With the addition of 5% molasses, the max LA (26.5-28.9 g/l) was achieved within 24 hrs and 48 hrs, respectively. In contrast, the addition of 10% molasses yielded more LA (48.7-50.2 g/l), but required a longer time (60 hrs) to reach this level. YE addition did not influence the Q_P value for formulations with 5% molasses. However, with 10% molasses, the Q_P value rose from 0.61 to 1.19 g/(l·h) with the quantity of YE added. The $Y_{P/S}$ ratio obtained in 5% molasses-enriched wheat stillage varied from 0.94 to 1.10 g/g, whereas that attained with 10% molasses enrichment was 0.67 to 1.43 g/g.

Control of pH with NH_4OH. In this experimental series (Table 6), the quantities of YE added were decreased (1.77, 3.54 and 7.08 g/l) but the enrichment of the medium with the carbon source (molasses, 5% and 10%) remained the same. The neutralizing agent for pH control was NH_4OH. It is interesting to note that in all of the experiments, the maximal number of the LAB supported by such media (0.9×10^{10} - 1.6×10^{10} cfu/ml) was higher than in the experiment with pH control using NaOH (1.2×10^{9}- 4.8×10^{9} cfu/ml) (Table 5). The max LA produced varied from 28.2 to 33.1 g/l (at 5% molasses enrichment), and from 39.9 to 49.8 g/l (at 10% molasses supplementation). At 5% enrichment, the quantity of YE added had no effect on the value of either $Y_{P/S}$ or Q_P. At 10% supplementation, the quantity of YE had some effect on Q_P and $Y_{P/S}$ (Table 6). The effect of YE addition on the theoretical yield was observed at 10% molasses supplementation. As the quantity of the YE added increased, so did the value of the theoretical yield. Regardless of the percentage of enrichment, molasses exerted an influence on both Q_P and $Y_{P/S}$. At 5% enrichment, Q_P values were lower (1.18 - 1.38 g/(l·h)) than at 10% enrichment (1.24-2.08 g/(l·h)). As for $Y_{P/S}$, the values obtained at 5% enrichment were higher (0.96 - 1.06 g/g) than those achieved at 10% supplementation (0.84 - 1.05 g/g).

DISCUSSION

These results demonstrate that the combination of wheat stillage medium, sugar beet molasses and YE may give good yields for cultivating lactic acid bacteria. The highest number of the LAB (1.6×10^{10} cfu/ml) was achieved by addition of 1.77 g/l YE to the sieved stillage medium containing 10% of molasses, when NH_4OH was used for pH adjustment (Table 6). The results also show that the proportion of molasses influenced the length of time over which the maximal number of LAB was achieved. At 5% enrichment, the LAB biomass took only 24 hrs to reach the maximal concentration. With twice the amount of molasses, the time to attain maximum LAB biomass also doubled. In molasses-enriched sieved stillage, media the maximal LAB concentrations were higher than in filtered stillage media (Table 3 and Table 5). This may be attributed to some key substances in the solids persisting in the sieved wheat stillage, which had the capacity to support bacterial growth. During our experiments, we achieved a higher amount of LAB (1.6×10^{10} cfu/ml) than did Chauhan et al. (2.34×10^{9} cfu/ml) after 48 hrs of *Lactobacillus* sp. KCP01 production in date juice supplemented with different components (Chauhan et al. 2007). The results of our present study show that wheat stillage supplemented with YE and molasses can be used as a medium for industrial scale production of lactic acid bacteria.

As for the growth of *L. plantarum* NC8 in hydrolysates of fish viscera, Horn et al. (2005) observed that media containing low amounts of YE or fish peptones produced biomass yields only slightly lower (by 10%) than the yield obtained with MRS medium. Horn et al. (2005) also suggest that the nitrogen sources in the MRS are present in excess, and that they are not assimilated by the biomass. Dumbrepatil et al. (2008) reported in their paper on LAB growth and LA production in YE-enriched cane

Table 5. Supplementation of sieved stillage with yeast extract (YE) and molasses (5% and 10%); pH controlled with NaOH.

Parameter	5%M			10%M		
	2.65g/l YE	5.31g/l YE	10.62g/l YE	2.65g/l YE	5.31g/l YE	10.62g/l YE
Glucose conversion [%] *	98.8	98.8	100	39.7	35.3	67.7
Max LA [g/l]	28.5 [36 h]	28.9 [48 h]	26.5 [24 h]	49.2 [60 h]	48.7 [60 h]	50.2 [60 h]
Maximal number of LAB [cfu/ml]	1.2×10^{9} [24 h]	2.7×10^{9} [24 h]	2.3×10^{9} [24 h]	2.2×10^{9} [24 h]	3.4×10^{9} [12 h]	4.8×10^{9} [12 h]
$Y_{P/S}$ [g/g] *	1.08 (108%)[a]	1.09 (109%)[a]	0.94 (94%)[a]	0.675 (27%)[a]	1.43 (50%)[a]	0.81 (54%)[a]
Q_P [g/(l·h)] *	1.11	1.04	1.10	0.61	1.14	1.19

*Glucose conversion, $Y_{P/S}$ and Q_P were calculated for values obtained in the 24 hrs of the process. [a]% of the theoretical yield.

molasses that the requirement of a low amount of YE as nitrogen source could be attributed to the fact that the quantity of nitrogen in the molasses was sufficient to support LAB growth. In our present study, YE addition to the molasses-enriched medium (5%) provided an almost complete assimilation of carbohydrates (expressed as glucose) after 24 hrs (Table 3, Table 5 and Table 6). Upon 10% enrichment with molasses, YE addition also produced a high extent of carbohydrates assimilation. However, for sieved stillage with pH controlled by NaOH, the proportion of carbohydrates assimilated over the same period was substantially lower (Table 5).

The neutralizer used for pH control also influences the maximum obtainable biomass of LAB. Results from our studies revealed that the amount of growth obtained for each culture neutralized with ammonium hydroxide was greater than that when sodium hydroxide was the neutralizer (Table 5 and Table 6). The same observation has been done by Gilliland who studied the effect of neutralizing agent on preparation of starter cultures of lactic streptococci (Gilliland, 1976). The higher amount of LAB obtained with NH_4OH as the neutralizer may have been due to the $NH4^+$ being stimulatory to the cultures. Peebles et al. (1969) suggested that the NH_3 may have raised the intracellular pH more rapidly than NaOH, thus allowing a more optimum pH for cell growth, resulting in a higher growth which was achieved in a shorter period. The ammonia could also serve as a nitrogen source for bacteria. It is possible that lower biomass amount observed when NaOH was used as neutralizer was due to the Na^+ being inhibitory (Peebles et al. 1969).

According to literature, sugar beet molasses typically consists of approximately 50% carbohydrates, where sucrose, glucose and other sugars account for 96%, 3% and 1.4%, respectively (Ghazi et al. 2006). The total sugar content of molasses used in our study was 53% w/v. In the medium with 5% molasses, total sugar concentration expressed as glucose equivalent was slightly higher than in 2% glucose medium, and approximately twice as high as in the 10% molasses medium (25.8 ± 0.8 g/l, 20 ± 0.2 g/l, and 53.4 ± 0.6 g/l, respectively). The optimum concentration of molasses in the medium could be somewhere between 5% and 10%. In our study the amount of molasses added was not optimized; it was adopted on the basis of relevant literature (Göksungur and Güvenç, 1999).

L. plantarum is known to be homofermentative for hexoses, producing 2 mol lactic acid per mol of hexoses (Passos et al. 1994). In our experiments using molasses-enriched media, we converted the yield of lactic acid into the glucose equivalent, to enable comparisons. The reason why the product obtained from the 2% glucose medium was lower compared with the one obtained from the 5% molasses medium (Table 2 and Table 4) could be that molasses contains also other components (*e.g*. other sugars, vitamins, amino acids, trace elements), which might enhance the yield of the product. In some of the processes, the actual yield of lactic acid was higher than the theoretical yield (Table 2 to Table 6). The explanation as to why the values of lactic acid yield were higher than those of the theoretical yield can be found in the utilization of other sugars (*e.g*. rafinose) that were present in the molasses. Another explanation was proposed by Ohmomo et al. (1988) who suggested that the LAB converted the melanoidins that were present in the molasses into lactic acid. Mussatto et al. (2008) report $Y_{P/S}$ ratios higher than 0.81 g/g, obtained with brewer's spent grain hydrolysate.

Table 6. Supplementation of sieved stillage with yeast extract (YE) and molasses (5% and 10%); pH controlled with NH_4OH.

Parameter	5%M			10%M		
	1.77g/l YE	**3.54 g/l YE**	**7.08 g/l YE**	**1.77 g/l YE**	**3.54 g/l YE**	**7.08 g/l YE**
Glucose conversion [%]*	100	100	100	51.1	82.7	99.8
Max LA [g/l]	33.1 [24]	28.4 [36 h]	28.2 [24 h]	39.9 [48 h]	47.6 [36 h]	49.8 [24 h]
Maximal number of LAB [cfu/ml]	1.3×10^{10} [24 h]	0.9×10^{10} [24 h]	1.3×10^{10} [24 h]	1.6×10^{10} [48 h]	1.3×10^{10} [48 h]	0.99×10^{10} [48 h]
$Y_{P/S}$ [g/g]*	0.96 (96%)[a]	1.06 (106%)[a]	1.02 (102)[a]	1.05 (53%)[a]	0.845 (70%)[a]	0.947 (95%)[a]
Q_P [g/(l·h)]*	1.38	1.18	1.18	1.24	1.55	2.08

*Glucose conversion, $Y_{P/S}$ and Q_P were calculated for values obtained in the 24 hrs of the process. [a] % of the theoretical yield.

These findings and reports have prompted us to further investigate the utilization of the carbon sources in the molasses-wheat-stillage medium by the same *L. plantarum* strain.

In summary, distillery wastewater and molasses may be used as nitrogen and carbon sources for large-scale fermentation of LAB, and thus help to avoid problems inherent in the use of animal-based nitrogen sources. The development of further media for LAB growth may eventually lead to a more efficient utilization of the waste streams from both distilleries and sugar mills.

CONCLUDING REMARKS

The results of this study suggest that wheat stillage, sugar beet molasses and yeast extract can be used as nitrogen and carbon sources for the production of *L. plantarum* on an industrial scale. The highest number of LAB (1.6×10^{10} cfu/ml) was achieved by the addition of 1.77 g/l YE to the 10% molasses-enriched sieved stillage, with NH_4OH used for pH adjustment. Wheat stillage contains a substantial fraction of bran (solids), which must be removed at a defined stage of the process if the LAB will be used as starter cultures for the food industry. In other applications, such as ensilage inoculation or animal probiotic, the solids might be left in the medium.

The use of a fermentation medium consisting of distillery wastewater and molasses to obtain value-added products (such as LAB biomass and lactic acid) is a possible step for classical ethanol production to move towards a biorefinery model production in which all by and waste products are utilized to increase produced values and reduce waste production. This enables a cost-effective utilization of the problematic wastewater from ethanol and sugar production.

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