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UTILIZATION OF WHITE GRAPE POMACE FOR LACTIC ACID PRODUCTION

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ABSTRACT
White grape pomace (WGP) contains high amount of soluble carbohydrates (glucose and fructose), which can potentially be used as a carbon source in the fermentative production of bio-based chemicals. In this study, this waste was valorized by lactic acid (LA) production by *Lactobacillus casei.* Adding WGP directly into the culture medium at a solid loading of 10% yielded 33.3 g/L LA. Water extract of WGP allowed comparably faster LA production. Increasing the solid loading in the extraction step increased the LA titers, however, it had a significant negative effect on the production rate. In all cases, fructose was consumed at a slower rate as compared to glucose. Yeast extract powder was required at a concentration of 10 g/L to support LA production. Alternatively, baker's yeast was autolyzed and the lysate was successfully used as the nitrogen source. The findings show that WGP can be regarded as a sustainable plant-based feedstock for LA production by *L. casei*, however, the probable negative effect of other pomace constituents should be avoided.
Keywords: grape pomace, lactic acid, autolysis, food waste valorization.

BEYAZ ÜZÜM POSASININ LAKTİK ASİT ÜRETİMİNDE KULLANILMASI

ÖZ
Beyaz üzüm posası (BÜP), biyo-temelli kimyasalların fermantasyonla üretimi için kullanılma potansiyeline sahip yüksek miktarda çözünmüş karbonhidrat (glikoz ve fruktoz) içerir. Bu çalışmada, bu atık *Lactobacillus casei* ile laktik asit (LA) üretilerek değerlendirilmiştir. BÜP kültür ortamına %10 oranında doğrudan eklendiğinde 33.3 g/L LA elde edilmiştir. BÜP'ün sulu özütü daha hızlı bir LA üretimi sağlamıştır. Özüt çıkarma aşamasında posa miktarının artırılması sayesinde daha fazla LA elde edilmiştir ancak, bu işlem üretim verimini kayda değer biçimde düşürmüştür. Tüm koşullarda fruktoz glikoza göre daha yavaş kullanılmıştır. LA üretimi için 10 g/L maya özütü tozu gerekmiştir. Buna alternatif olarak, ekmek mayası otolize uğratılmış ve bu lizat azot kaynağı olarak başarıyla kullanılmıştır. Bulgulara göre, BÜP'ün *L. casei* ile LA üretimi için sürdürülebilir bir kaynak olduğu düşünülebilir, ancak, posada bulunan diğer maddelerin olası olumsuz etkileri önlenmelidir.
Anahtar kelimeler: üzüm posası, laktik asit, otoliz, gıda atıklarının değerlendirmesi.

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INTRODUCTION

There is an increasing interest in bio-based chemicals and materials, which are recovered or produced from biological materials. Food, agricultural and some other industries release huge amount of waste and by-products, most of which has little or no economic value. Lignocellulosic waste biomass, such as straw, bran, stalk, stover, cob, shell, wood, saw dust, and bagasse are abundant potential feedstocks, however energy intensive and costly pretreatment processes are required for their utilization in bioprocesses (Zabed et al., 2016). Fruit wastes, on the other hand, contains easily fermentable monosaccharides as well as structural cellulose, hemicellulose, and pectin. For example; Choi et al. (2015) reported high fermentable carbohydrate content (sum of glucose, fructose, and sucrose) in the peels of orange (53.2%), mandarin (57.1%), apple (59.1%), grapefruit (43.2%), and banana (45.3%) on dry basis. Unlike cellulose and hemicellulose in the lignocellulosic biomass, fermentable carbohydrates can be utilized by microorganisms for growth and product formation without the need of a pretreatment.

Similar to some other fruits, grapes (*Vitis vinifera* L) contain substantial amount of soluble carbohydrates, which are the substrates for yeast growth and ethanol production during wine production. These carbohydrates are made available to wine yeast by breaking berries to release juice (crushing). Pomace is separated from red wine after the fermentation step. On the other hand, in white wine production skins are removed by pressing after crushing and juice is used as the fermentation medium. As a result of that, and due to using different grape varieties, the pomaces from red and white wine productions have different compositions (Deng et al., 2011). Red grape pomace is rich in dietary fibers and phenolic compounds (Deng et al., 2011, Xu et al., 2016), whereas WGP contains high amount of water soluble carbohydrates (Deng et al., 2011, Zheng et al., 2012). Therefore, WGP can be considered as a convenient source of carbon for microbial processes. Carreira et al. (2011) produced bacterial cellulose from the soluble carbohydrates in the aqueous extract of WGP. Mendes et al. (2013) produced bioethanol from the aqueous extract of WGP, and in addition to that, recovered oleanolic acid in the organic extract and used remaining solids to manufacture low-density board.

Annual global grape production was 74.5 million tons in 2014 (FAO, 2014). Eighty percent of the all grapes is used in wine making (Fontana et al., 2013), thus wine industry discards huge amount of grape skin, stem, seeds, and lees. Approximately 20% of the grapes are discarded as pomace, which can be used as fertilizer and animal feed. However, high levels of phenolic compounds limit the utilization of pomace as fertilizer, while in animal feeding, lignin content reduces the digestibility of the pomace (Fontana et al., 2013). Pomace polyphenols and seed oil are valuable as health and food products ingredient (Korkie et al., 2002, Zheng et al., 2012).

Lactic acid (LA) and its salts have long been used in food and pharmaceutical industries and more recently there is an interest in poly-LA, which is a biodegradable polymer produced from optically pure LA monomer (Castillo Martinez et al., 2013). LA has also a high potential as one of the platform chemicals that can be derived from biomass resources (Dusselier et al., 2013). There are many efforts to produce this chemical from waste materials in cost effective and environmentally friendly processes (Wang et al., 2015, Kawaguchi et al., 2016). In this study, the potential of white wine grape pomace for LA production was assessed. The aim was the valorization of a food industry waste and development of a low-cost fermentation medium for an industrially valuable chemical.

MATERIALS AND METHODS

Materials

White grape pomace (WGP) from Muscat variety was obtained from Urla Winery (İzmir) in September 2012 and kept at -20°C until use. WGP was dried in an oven (Termal, İstanbul, Turkey) at 60°C for 24 h. After drying, seeds and stems were removed and the rest was ground to a fine powder with a kitchen grinder. All chemicals were of analytical grade and purchased from Sigma-

Aldrich (Steinheim, Germany) and Merck Millipore (Darmstadt, Germany), except baker's yeast (Pakmaya, İzmit, Turkey), which was purchased at a local market. *Lactobacillus casei* NRRL B-441 was kindly provided by United States Department of Agriculture, National Center for Agricultural Utilization Research.

Yeast Autolysis

Commercial baker's yeast (12 g on dry basis) was put in a 250 mL flask and distilled water was added to a final volume of 120 mL. pH was adjusted between 5-7 and the flask was incubated in a shaking incubator (Zhicheng, Shanghai, China) at 100 rpm, at 50 °C for 48 h (Şahin, 2002). After the incubation period, solid cell debris was removed by centrifugation and supernatant (autolysate) was added to the LA production medium.

Lactic Acid Production

L. casei was maintained in De Man-Rogosa-Sharpe (MRS) broth, which was composed of (g/L): peptone from casein (10.0); meat extract (10.0); yeast extract (4.0); D-(+) glucose (20.0); K_2HPO_4 (2.0); Tween 80 (1.0); di-ammonium hydrogen citrate (2.0); sodium acetate (5.0); $MgSO_4$ (0.20); $MnSO_4$ (0.04). The MRS broth and all other growth and fermentation media were sterilized at 121 °C for 15 min in an autoclave (Hirayama, Tokyo, Japan).

Unless otherwise indicated, LA production medium was composed of (g/L): yeast extract (10); K_2HPO_4 (0.50); $MgSO_4$ (0.20); $MnSO_4$ (0.05); and the carbon source. In one set of experiments dry WGP was added to the medium without any pretreatment. In the other set, WPG was first extracted in hot water at 80 °C for 60 min in a water bath (Termal, İstanbul, Turkey) and the other ingredients were added to the extract after removing the remaining solids by centrifugation (Centurion, West Sussex, UK). Analytical grade glucose and fructose were used as the carbon sources in the control run. Seventy mL of the production medium was put in a 250 mL flask and the flask mouth was covered with aluminum foil before sterilization. $CaCO_3$ powder was sterilized separately and added before inoculation (1g $CaCO_3$ per flask) in order to maintain pH level at 5-6 during the process. The production medium was inoculated (1% (v/v)) from 24 h old culture, which was grown in MRS broth at 37 °C in a static incubator (Termal, İstanbul, Turkey). Flasks were incubated in a shaking incubator at 37 °C and 150 rpm. Samples (2 mL) were taken at intervals and centrifuged at 3024 *g* to remove pomace, cells and undissolved $CaCO_3$. The supernatants were kept at -20 °C until HPLC analysis.

Analytical Methods

Cellulose and hemicellulose contents of the pomace were measured indirectly using two-stage acid hydrolysis. In this method, cellulose and hemicellulose was hydrolyzed to corresponding monomers, which were then quantified in HPLC. In the first stage, dried pomace (300 mg) free of stems and seeds was suspended in 3 mL of 12 M H_2SO_4 in a test tube and the tube was kept at room temperature for 3 h. In the second stage, water was added to the suspension to a final H_2SO_4 concentration of 0.80 M and the tube was kept at 100 °C in the water bath for 4 h (Zhou and Ingram, 2000). The pH was neutralized by $CaCO_3$ and insoluble materials were removed by centrifugation at 3024 *g*. Supernatants were diluted with water and analyzed for glucose, xylose, and arabinose and the concentrations were multiplied by anhydro factors (0.90 for glucose; 0.88 for xylose and arabinose) to calculate cellulose and hemicellulose contents of the pomace, respectively.

WPG hot water extract and the samples from LA production were analyzed for carbohydrates and LA. Samples were diluted at least 30 times with water in order to decrease the sugar and LA concentration below 1 g/L.

Glucose, fructose, xylose, arabinose, and LA contents were measured by HPLC (Perkin Elmer, USA) using Aminex HPX-87H column (Biorad Laboratories, CA, USA) operated at 65 °C and a refractive index detector. The mobile phase was 5 mM H_2SO_4 flowing at a rate of 0.6 mL/min. The peak areas in the chromatograms were used to

calculate the concentrations of the analytes by comparing to respective calibration curves.

RESULTS AND DISCUSSION

Carbohydrate Content of Grape Pomace

Free and easily fermentable carbohydrate content of the WGP was of great importance in this study, since in bioprocesses, the amount of target product depends on the initial substrate concentration as well as the conversion capacity of the fermenting microorganism. The potential of WGP as a fermentation substrate was determined by quantification of the available carbohydrates after a mild extraction step. Extracting WGP in water at 80 °C for 60 min yielded 18.7% glucose and 17.7% fructose (based on dry weight of WGP). Both glucose and fructose contents were in good agreement with the ones observed in the pomace from Sauvignon Blanc (Corbin et al., 2015), whereas higher total soluble sugars were reported in Muller Thurgau (56%) and Morio Muscat (78%) pomaces (Deng et al., 2011), and in a fresh grape pomace (49%) (Zheng et al., 2012). The results showed that WGP could provide substantial amount of easily fermentable carbohydrates and had a potential to be used as carbon source in fermentative production of bio-based products. Glucose and fructose in WGP can be extracted and added to the production media after the solid part is removed. Alternatively, GP can be used as a carbon source without a pre-extraction step or any other treatment. Both alternatives were tested in this study.

The structural polysaccharides in various wastes are potential carbon sources for fermenting microorganisms provided that they are hydrolyzed into fermentable monosaccharides. Therefore, the cellulose and hemicellulose content in WPG was quantified after acid hydrolysis. WPG was found to contain 10.6% cellulose and 3.41% hemicellulose. These results were in good agreement with those obtained in previous studies (Zheng et al., 2012, Corbin et al., 2015). The soluble carbohydrate content of WPG used in this study was approximately three times the insoluble polysaccharide content. Similarly, Corbin et al. (2015) reported that pomace of Sauvignon Blanc contained 43% soluble carbohydrates, which value was almost four times the amount of the insoluble polysaccharides. On the other hand, lignocellulosic biomasses, such as agricultural wastes, generally contain higher amounts of structural polysaccharides (Botella et al., 2005, Menon and Rao, 2012). Taking into account the low polysaccharide content in WGP and the high cost of pretreatment needed to convert those polysaccharides into fermentable monosaccharides, in this study, only the soluble carbohydrates were utilized for LA production.

Lactic Acid Production from Glucose and Fructose

One of the problems related to using complex substrates, such as food waste, in growth and production media is their unknown composition (Hayek and Ibrahim, 2013, Basu et al., 2015). They may include compounds that impair the growth of the fermenting microorganism or inhibit the formation of the target product. Considering this, the capacity of *L. casei* for LA production was tested first in a medium containing purified (analytical grade) glucose and fructose (control run). This information was used in the following steps to evaluate the cultures in which WGP was used as the carbon source. The initial concentration of each monosaccharide was adjusted to 20 g/L, which simulated the carbohydrate profile of a medium containing 10% WGP (w/v), and the carbohydrate consumption and the LA production were followed (Figure 1). Glucose was utilized rapidly and depleted in 24 h, while fructose consumption was slower as compared to glucose. When fructose was used as the sole carbon source at a concentration of 20 g/L, its consumption was faster (data not shown) compared to the mixed substrate case. This showed that, the presence of glucose exerted a partial inhibition on fructose utilization. Nancib et al. (2009) observed a similar trend in LA production from date juice extract by *L. casei* and *Lactococcus lactis*. Both bacteria utilized glucose more rapidly than fructose in the early stage of the cultures. Similarly, during cucumber fermentation by *L. plantarum*, glucose was consumed slightly faster than fructose (Lu et al., 2001).

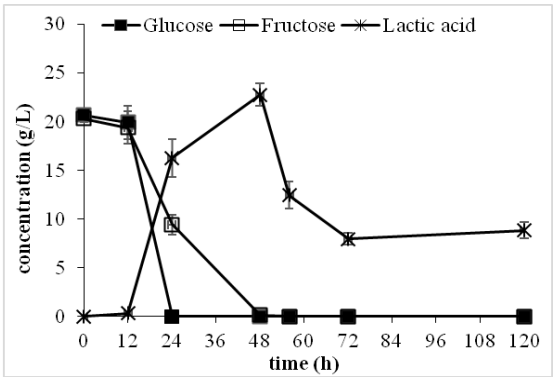

Figure 1. Carbohydrate consumption and lactic acid production from analytical grade glucose (20 g/L) and fructose (20 g/L).

Lactic Acid Production from White Grape Pomace

Direct utilization of a waste material in a bioprocess is advantageous due to not requiring a costly pretreatment step. Therefore, in the first attempt for utilization of WGP as the carbon source, dried WGP was added to the culture media (10% w/v) without applying any treatment. The initial concentrations of glucose and fructose were measured as approximately 20 g/L (Figure 2). Interestingly, the concentrations of these monosaccharides increased in the first 4 h, which could have been due to release of carbohydrates trapped in the pomace. This may have been possible owing to the bacterial action or agitation. After the rise in the early stage, the glucose concentration decreased following almost a linear trend and depleted in 48 h. Fructose was consumed at a slower rate similar to the trend observed in the control run, in which *L. casei* was grown on analytical grade glucose and fructose. The maximum LA concentration (33.3 g/L) was observed after 72 h, at which point there was not any soluble carbohydrates left in the culture medium (Figure 2). Taking into account the carbohydrate concentrations after first 4 h, LA yield was calculated as 0.58 (g LA)/(g carbohydrates), which value was very close to the one in the control run. On the other hand, the maximum LA production rate (0.60 g/(Lh)) obtained with WGP as the carbon source was almost half of the rate in the control run. The lower production rate was apparently due to the slower utilization of carbohydrates. This behavior could be attributed to the contents of the pomace, which may have impaired the growth of *L. casei* or its carbohydrate consumption, or both. Some compounds that were toxic to the bacteria may have been extracted from pomace during sterilization step at 121 °C or during cultivation. Phenolic compounds, such as wine related ones, have been reported to have an effect on growth or viability of bacteria including *Lactobacillus* species, such as *L. hilgardii, L. plantarum,* and *L. casei* (Garcia-Ruiz et al., 2011, García-Ruiz et al., 2012, Sabel et al., 2017).

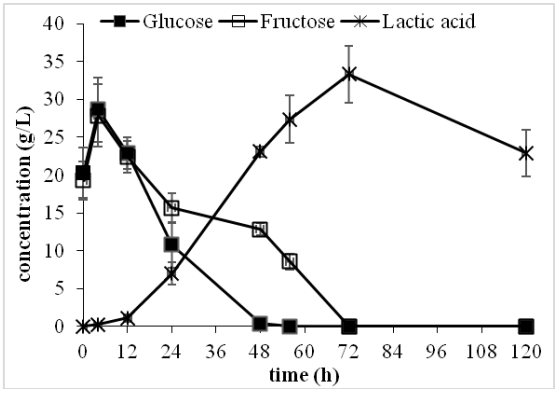

Figure 2. Carbohydrate consumption and lactic acid production from untreated white grape pomace (10% solid loading).

Lactic Acid Production from White Grape Pomace Extract

The probable negative influence of grape pomace on the fermenting organism can be avoided by preventing the release of compounds from the pomace and/or avoiding the contact between the bacteria and the pomace. In this study, these were achieved by pre-extraction of the pomace in water to release soluble carbohydrates and removal of the solid material by filtration afterwards. This way, pomace did not encounter high sterilization temperature (121 °C), so that release of compounds, such as phenolic compounds, may have been limited. In addition to that, *L. casei* did not contact the pomace during cultivation, since it was grown in the extract, which was free of solid pomace material.

The WGP was treated with hot water at a solid to liquid ratio of 1:10 at 80 °C for 60 min. The other nutrients were added to the extract and this medium was used for LA production. In this culture, glucose was utilized more rapidly compared to the cultures mentioned above. After 12 h, no glucose was detected in the culture medium (Figure 3A). Fructose consumption was slower than glucose, similar to the previous cases, and it was depleted in the medium in 48 h. At this time point, LA concentration was 22.1 g/L, while after 60 h, slightly higher amount of LA (24.1 g/L) was detected. In the first 12 h, LA production rate was considerably high in parallel to rapid glucose consumption. In this phase, 17.2 g/L LA was produced at a rate greater than 1.4 g/(Lh). After that time point, when fructose was the sole carbohydrate, its consumption and consequent LA production was much slower. During this phase, 6.9 g/L LA was produced in 48 h, corresponding to 10-fold lower production rate as compared to the initial phase. The total yield based on LA concentration after 60 h was calculated as 0.61 (g LA)/(g carbohydrates), while production rate was 0.40 g/(Lh).

In the next attempt, the pomace loading in the extraction step was increased to a solid to liquid ratio of 1:6.7 in an effort to obtain a more concentrated extract. As a result, LA production process started with increased amount of glucose and fructose (Figure 3B). Glucose utilization rate was only slightly slower than the previous case and it was depleted between 12 and 24 h of fermentation. Unlike the cultures above, in which fructose was consumed in parallel to glucose albeit at lower rates, in this case *L. casei* did not start to use fructose before glucose was consumed entirely. Fructose utilization started after 24 h and the utilization rate was relatively slow. In the first 24 h, 20.7 g/L LA was produced at a rate of 0.86 g/(Lh). In the second phase of the culture, 19.4 g/L LA was produced from fructose in 108 h, corresponding to LA production rate of 0.18 g/(Lh). Fructose utilization was completed after 132 h, at which point LA concentration was 40 g/L. Accordingly, the total LA yield was calculated as 0.75 (g LA)/(g carbohydrates), which value was 23% greater than the one obtained in the previous run. On the other hand, the overall LA production rate (0.30 g/Lh) was 25% lower. The high solid loading in extraction yielded more LA as a result of higher initial carbohydrate concentration in the medium, however, the productivity was lower due to the late onset and low rate of fructose consumption. Consequently, it was possible to increase the product concentration and yield only by compromising from production rate.

The slow fructose utilization and LA production in the latter culture was probably not related to the relatively higher carbohydrate concentration, since *Lactobacillus* species were reported to function efficiently under high carbohydrate levels (Abdel-Rahman et al., 2013). *L. casei* was reported to utilize date juice containing glucose (50-55 g/L) and fructose (40 g/L), and produced over 53 g/L LA in 19 h (Nancib et al., 2009). It also could produce LA at a rate of 2.2 g/(Lh) from reconstituted whey powder with a lactose concentration of 103 g/L (Buyukkileci and Harsa, 2004). Thus, the impaired LA production was probably due to the presence of other compounds in the medium. During extraction, some chemicals that were potentially toxic to *L. casei* could have been released, similar to the case in which WGP was added directly into the medium. This negative effect was not experienced in the previous culture where the solid loading in the extraction was low, so that the toxic compound concentration in the extract may have been below a critical level.

Effect of Yeast Extract and Yeast Autolysate

Lactobacillus species are fastidious in their nutritional requirements and require some amino acids and vitamins in the growth media (Hofvendahl and Hahn–Hägerdal, 2000). The most common source of these has been yeast extract, which provides nutrients that most microorganisms may need for their growth (Rivas et al., 2004). Since using yeast extract increase the cost of LA production media, some other nitrogen sources have also been used to support the growth of *Lactobacillus* species (Abdel-Rahman et al., 2013, Ghaffar et al., 2014). In this study, the effect of yeast extract concentration was tested

and next, autolyzed baker's yeast was used in the LA production medium containing 10% (w/v) WGP.

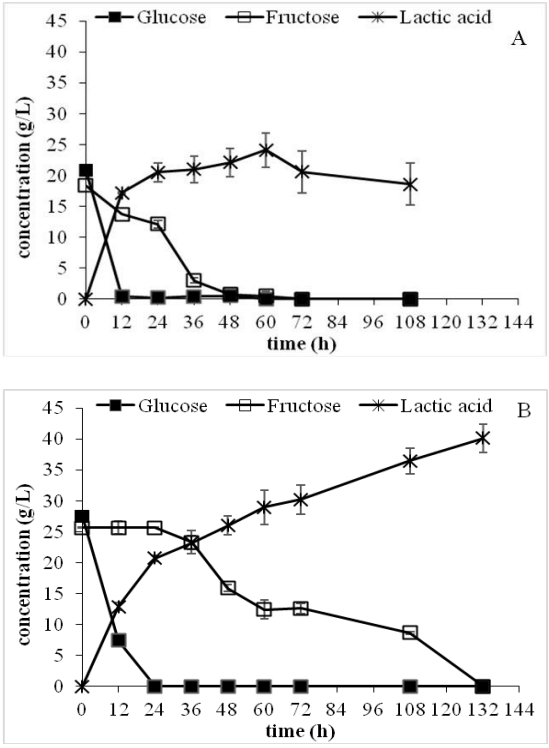

Figure 3. Carbohydrate consumption and lactic acid production from white grape pomace extracts with extraction solid to liquid ratio (w:v) of A. 1:10 and B.1:6.7.

When yeast extract concentration was reduced to 5 g/L, as opposed to 10 g/L in the previous runs, the organism grew only poorly. On the other hand, using 15 g/L of yeast extract did not improve the LA production as compared to using 10 g/L, resulting in almost the same fermentation kinetics (data not shown).

In the next step, commercial baker's yeast (*Saccharomyces cerevisiae*) was autolyzed and added to the LA production medium containing WPG, instead of using yeast extract powder. Autolysis is the breakdown of cellular components by the action of the hydrolytic enzymes found within the cell. As a result, biopolymers in the cell, such as proteins, are hydrolyzed into low molecular weight products and released (Bustos et al., 2004). In previous studies, yeast autolysate was used successfully as a nutrient for *Lactobacillus* species (Borzani et al., 1993, Michelson et al., 2006, Bolner de Lima et al., 2009).

In this study, the liquid lysate obtained by autolysis of baker's yeast was introduced to the medium at two levels, namely 10 mL and 25 mL, while keeping the working volume (70 mL) and WPG loading (10%) constant. In both cases, similar LA concentrations were observed (33.1-33.5 g/L) at the end of the fermentations (Figure 4A and 4B), which levels were also very close to the ones obtained with yeast extract powder (Figure 2). The low lysate dose could support the growth and LA production; however, notable carbohydrate utilization and LA production did not start before 24 h (Figure 4A). After 72 h, glucose was depleted, fructose was low and LA production was completed. The high lysate dose, on the other hand, yielded faster fermentation, thus glucose consumption and LA production were rapid, while efficient fructose utilization began after 24 h (Figure 4B). In this case, fermentation was completed in 48 h. The results showed that autolysis of baker's yeast could provide the nutritional requirements of *L. casei*, thus could be used as an alternative to yeast extract powder. Instead of baker's yeast, it may be possible to use waste yeast obtained after the wine fermentations.

Utilization of food waste in the fermentation medium without any energy intensive pretreatment processes is a cost-effective solution for providing fermentable carbohydrates for the microorganisms. WPG was successful in supporting the growth of *L. casei* and its LA production, either in untreated form or after a simple hot water extraction step. It was also possible to supply the other nutritional requirements of the bacteria by autolysis of baker's yeast. Thus, the growth medium used in this study can be considered sustainable for production of LA or other bio-based products. WGP or its extract concentration was limited due their negative effect on the culture performance. Relating this to the toxic effect of phenolic compounds, a pre-extraction step to recover these

compounds not only can diminish the negative effect, but also provide functional ingredients for food and pharmaceutical industries.

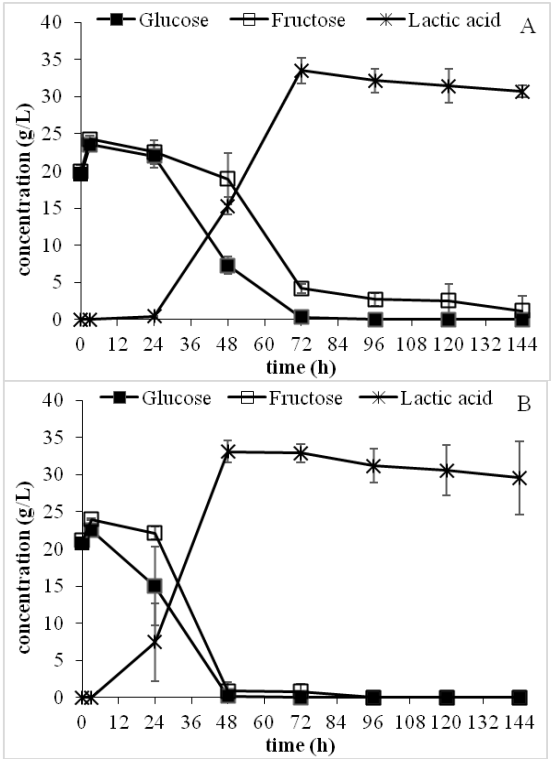

Figure 4. Effect of yeast autolysate as the nitrogen source for lactic acid production from untreated white grape pomace (10%). A: 10 mL, B: 25 mL yeast autolysate in total medium volume of 70 mL.

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