PRODUCTION OF *β*-GLUCAN FROM WINERY YEAST WASTE BIOMASS

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Abstract. The aim of this study was dual. Firstly, the exploitation of a completely new and alternative source for yeast *β*-glucan industrial production, yeast wastes biomass. Secondly, the utilization of winery yeast wastes for the production of added value products as a part of an intergraded and environmental friendly wine industry. *β*-Glucan isolation from red wine lees was performed in a two step process, yeast autolysis and alkali extraction with NaOH. Autolysis at 55^{o}C/pH 5.0/24 h with the use of natural acetic acid for the production of a natural yeast extract was optimum. Most purified fraction of *β*-glucan (19.56 %) was received with mild NaOH 0.25 M/1.5 h/90^{o}C. The highest yield (6.34 %) was obtained with NaOH 1 M/0.5 H/90^{o}C. Winery yeast waste lees can comprise an innovative way for *β*-glucan production. Further research in extraction methods, immunoactivity and applications of obtained *β*-glucan from this source, must be investigated. Also, a deeper research for a new *β*-glucan with wine polyphenols product for incorporation in functional foods and medicines is proposed.

1. Introduction

The winemaking process is leading to the formation of a by-product called wine lees. Wine lees are formed during the wine fermentation, filtration, centrifugation, and aging steps for the wine production [1]. The main volume of them is generated before and after the completion of alcoholic and malolactic fermentation (fig. 1). These are a sludge remaining as a residue at the bottom of fermentation tanks. Wine lees consist

mainly of spent yeasts and secondary of bacteria, phenolics and pigments (red wines), tartaric acid and ethanol [2]. The major structural elements of yeast cell wall are mannoproteins (c.a. 35-40 % of cell wall) and β-glucans (c.a. 50-55% of cell wall). The minor components are α-glucan, chitin, proteins and lipids [3]. Yeast β-glucan molecule is a D-glucose biopolymer. In yeast cell wall two different types of β-D-glucans are found: these are β-1,3-D-glucan, a main component (85%) of about 1500 residues, representing more than 50-55% of cell wall and β-1,6-D-glucan amounts (15%) of about 140 residues, representing 5-10% of cell wall [4,5]. β-Glucan is a widespread molecule in the cell wall of many organisms: yeast, bacteria, fungi, algae and plants. The yeast cell wall accounts 20-30% of cell dry mass [6].

β-Glucans are called biological response modifiers (BRMs) due to their ability of enhancing and stimulating the human immune system. They have been proved beneficial for various human and animal diseases and disorders [7,8]. β-Glucans obtained from the cell wall of yeasts can also be used in food industry as fat replacers, emulsifiers and dietary fibers [9]. Yeast β-glucan extract is considered as safe for oral applications and recognized as GRAS (Generally Recognized As Safe) [9].

For the β-glucan production, yeast cell walls must be prepared. These are obtained after the yeast cell is lysed or disrupted and the cell cytoplasm flows out. The yeast cell lysis disruption is achieved with physical (sonication, homogenization), chemical (alkali, acid) and/or enzymatic (lytic enzymes, glucanases) procedure [10-12]. In a subsequent step, the obtained yeast cell walls have to be purified. At the beginning the mannoprotein and then the lipids and proteins are removed, leading to different fractions of purified β-glucan. β-Glucan extraction and purification methods are based on alkali and acid hydrolysis, enzymes and/or sonication treatment [13-20].

The production of β-glucan from breweries spent yeast by-product has been already achieved and the prepared glucan is proposed for use as an ingredient, an additive, an animal diet supplement, a fish immunostimulant, and a BRM immunoactivator [21-29]. Yeast lees from winery waste have not been yet exploited for β-glucan production [30]. In winery industry, the sludge remaining after the alcoholic fermentation contains a significant amount of spent yeast which with the appropriate biotechnological methods can give added value products such β-glucans. Instead of this, the most common practise is the disposal and landfill of these wastes causing environmental damage [2, 30, 31].

In this study, we propose a two-step method based on yeast autolysis and hot alkali for β-glucan extraction from a new and unexploited source, winery yeast waste biomass. The purpose of this paper is to offer the winery industry new products as well as to apply a method for the treatment of a waste helping the environment.

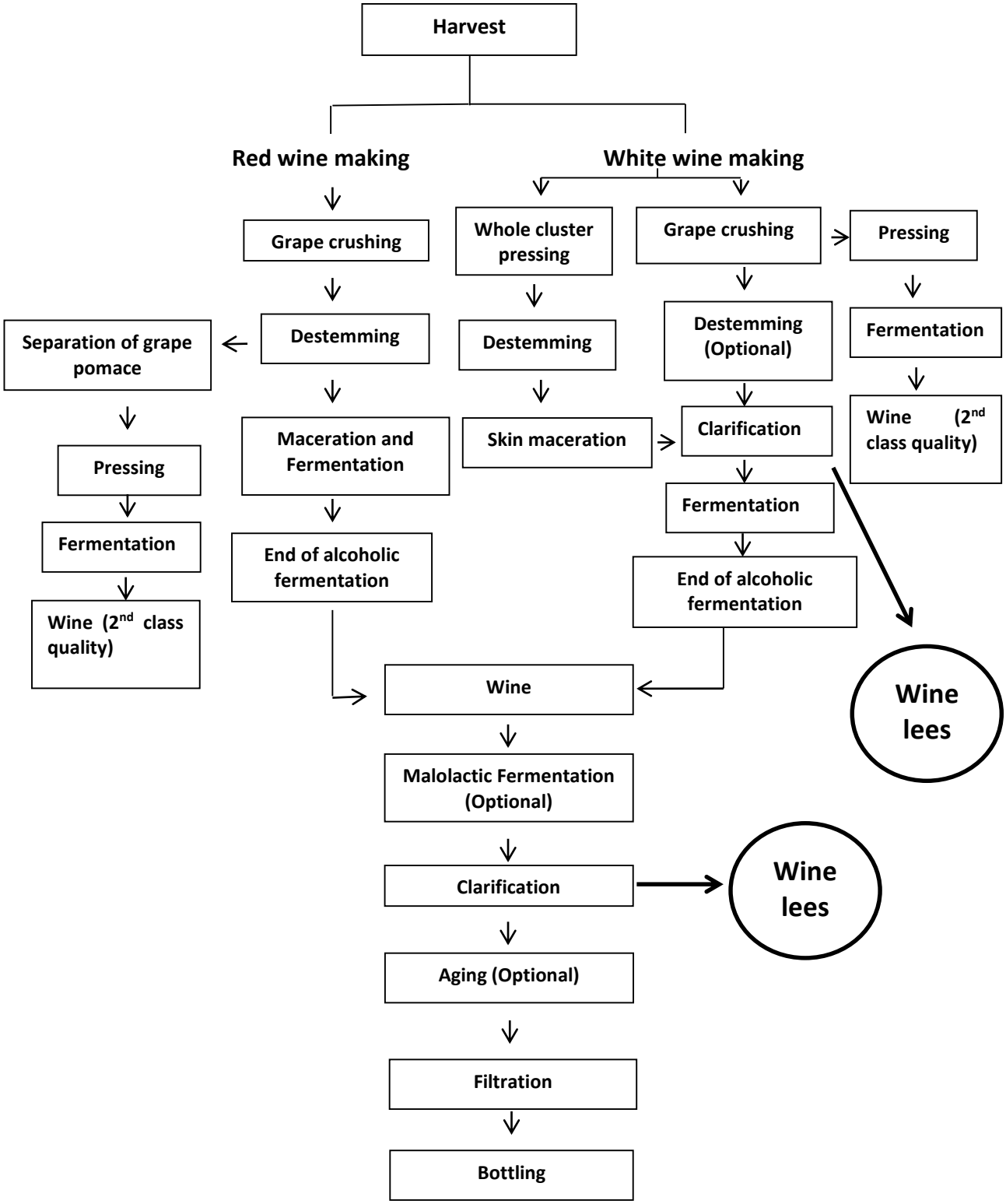

Fig.1. Red and White wine making process and the steps where the major volume of yeas lees waste is generated

2. Materials and methods

2.1. Materials

2.1.1. Commercial dry yeast

Yeast cells VIN 13 *S. cerevisiae* strain were provided by Anchor.

2.1.2. Winery spent yeast lees

Wine yeast lees were provided by Nemea region (Lantides Estate) and Ritsona region (Logothetis Winery). The wine lees were collected after the completion of alcoholic fermentation for red wine and the clarification for white wine production. The liquid supernatant (wine) was analyzed for the determination of various enological parameters. The liquid supernatant obtained after the clarification of white wine must, was used for the preparation of natural acetic acid. The wet sediment of red wine yeast lees, was used for *β*-glucan isolation.

2.1.3. Chemicals

NaCl, NaOH, and all reagents used, were of analytical grade

2.2. Methods

2.2.1. Determination of water and total solids of wine lees

The wine lees samples were homogenized by stirring. Then, 5 ml of each sample was collected and dried at 103°C for 24 hr. The water content and total solids were determined gravimetrically [32]

2.2.2. Determination of enological parameters of wine

The finished wine was analyzed according to OIV's official methods for the determination of pH, alcohol, total acidity and volatile acidity. The other parameters were determined with the use of WineScan apparatus (Foss Co.).

2.2.3. Determination of β-glucan concentration

The determinations of total yeast *β*-glucan concentration in extracted yeast powder were performed with the use of K-EBHLG Enzymatic Yeast Beta-Glucan Assay Kit (Megazyme, Ireland).

2.2.4. Determination of total phenolic compounds

The extracted phenolic compounds were determined with Folin-Ciocalteu method according to the procedure proposed by Chatzilazarou et al. [32]

2.3.Procedures

2.3.1. Yeast autolysis-cell wall preparation

100 g of dry yeast VIN 13 were divided in two equal portions (each of 50 g) and then diluted to dionized water leading to yeast cells slurry of 10% and 20% (w/v) respectively. The pH was brought to 5.0 with natural acetic acid and 3% NaCl was added as the autolysis promoter. The acetic acid was produced after acetic acid bacteria fermentation on the liquid supernatant of white wine yeast lees produced at must clarification stage. The mixture was incubated at 55^{o}C for 24 h with mild agitation. Then, the autolysate was heated at 80^{o}C for 15 min for the deactivation of the endolytic enzymes. The yeast extract (supernatant) was separated from autolyzed yeast cells (sediment) with centrifuging (5000 rpm/10 min). The autolysis ratio was calculated as follows: 5.0 ml from the initial yeast slurry and 5.0 ml from the autolysate were dried and the dried biomass was weighted. The autolysis ratio (R) is the loss of dried biomass before (Wo) and after autolysis (W) [17, 32]. Yeast red lees were diluted to deionized water leading to yeast cells slurry of 10% and then were subjected to autolysis procedure as above. The autolyzed yeast was stored at 4^{o}C for further treatment.

2.3.2. Extraction of yeast β-glucan from VIN 13 S. cerevisiae strain

Yeast VIN 13 dry mass obtain from autolysis procedure was diluted in 0.25, 0.5 and 1 M NaOH leading to yeast cell slurry of 10% and 20% (w/v). The content was heated at 90^{o}C under stirring for 1.5 h. The supernatant was discarded and the sediment was lyophilized for *β*-glucan content determination.

2.3.3. Optimization of alkaline extraction of yeast β-glucan from red wine waste lees

2.3.3.1. Optimization of NaOH concentration.

For each yeast lees sample, 10 g of yeast waste biomass was diluted in 0.25 M, 0.50 M, 0.75 M and 1 M NaOH (20% w/v). The content was heated at 90^{o}C under stirring for 2 h. The supernatant was discarded and the sediment was lyophilized for *β*-glucan content determination.

2.3.3.2. Optimization of extraction time

At extraction time 0.5 h, 1.0, 1.5 h and 2.0 h a sample of 10 ml for each NaOH concentration was collected and centrifuged (5000 rpm/10 min). The supernatant was analyzed with Folin-Ciocalteu method for polyphenols content determination and the sediment was used for *β*-glucan isolation with the same procedure as for optimization of NaOH concentration. Also, samples of 10 ml from yeast lees before and after induced autolysis were collected, dried and used for *β*-glucan determination.

2.3.4. Lyophilization and weight of dry prepared β-glucans

The sediment of *β*-glucans was frozen and then lyophilized (-80^{o}C/vacuum/24h) using a Thermo Fischer (USA) drying digital unit. The weight of dried *β*-glucan of each sample was measured with an analytical balance (Kern, Kern and Sohn GmbH) with an accuracy of four decimals.

2.3.5. Yield calculation of produced β-glucan

The yield of produced *β*-glucans was calculated as the percentage of dry weight of wet *β*-glucans from dry weight of the yeast lees starting material

2.3.6. Statistical analysis

Each experiment was repeated three times. Results are displayed as means of three determinations of three simultaneous assays in all methods. Experiments were set up in a completely randomized design and results were assessed by a standard analysis of variance.

3. Results and discussion

3.1. Yeast autolysis

Autolysis is a procedure by which yeast cell is self-lysed by the release of cell lytic enzymes into the cytoplasm by the cell lysosomes [13]. The whole process is defined as the hydrolysis of intracellular biopolymers under the effect of hydrolytic enzymes. The autolysis is a procedure for yeast cell wall preparation under mild conditions [14, 17]. The duration of the process is long and lasts from 24 h until some days [13]. Cell concentration, pH, autolysis promoter, temperature and duration are the parameters that affect the yeast cell autolysis ratio [17]. The VIN 13 yeast cell and yeast lees cell autolysis ratio was tested according to pH, temperature and duration of autolysis procedure for the determination of the optimal conditions for yeast cell autolysis and preparation of yeast cell walls.

The optimal autolysis conditions for VIN 13 commercial yeast and winery yeast lees were addition of 3% NaCl as an autolysis promoter, pH 5.0, incubation in water bath at 55^{o}C for 24 h. The pH was set to 5.0 with the use of natural acetic acid and not with HCl as others researchers report [14, 17]. The produced autolysate was a natural product without the use of chemicals or enzymes. Under these conditions, the autolysis ratio (R%) was 27.42 % and 23.38 % for yeast cell slurry 10% and 20% concentration respectively. These values differ from values reported from other researchers under the same conditions. However, the differences maybe arise from the different strain that these researchers used [14, 17] and the conditions under which this stain was cultivated [6]. Also, the differences from other research works can be explained by the use of brewery spent yeast and not winery one [17]. The autolysis ratio of yeast lees was 14.4 %. This can be explained by the fact that at the end of the wine fermentation process, a significant number of yeast cells undergo to enzymatic cell wall degradation autolysis and release of polysaccharides. Due to that, spent yeast cells after fermentation process are used for the production of wines aged on yeast lees [34].

3.2. Yeast β-glucan extraction

3.2.1. VIN 13 S.cerevisiae strain

The yeast cell walls obtained from the autolysis process were subjected to alkaline treatment with NaOH for the removal of mannoproteins from the outer layer of the yeast cell wall [26]. From VIN 13 strain, six different *β*-glucans were prepared. Before alkaline treatment yeast cells slurry in 10% and 20% (w/v) was prepared. This was done for the estimation of the appropriate yeast cell concentration which is leading to the most extractable portion of *β*-glucans. For alkaline extraction, two different NaOH concentrations in both prepared yeast slurry were tested for the optimal concentration of NaOH for mannoprotein removal and *β*-glucan preparation. This was done for comparative results in NaOH concentration used for *β*-glucan preparation from pure commercial dry yeast which has not been exposed to the stressed conditions of fermentation process for wine making as yeast by-products of the fermentation process. The extraction time with NaOH for all samples was 1.5 h. The 20% (w/v) cell suspension with 0.5 M NaOH for 1.5 h at 90^{o}C gives the highest *β*-glucan concentration (58.36 ± 1.58 %) in the final yeast powder obtained from the cell wall of VIN 13. From the results it is also pointed that the initial cell suspension plays an important role not only during autolysis process for cell wall production but also at the *β*-glucan preparation with NaOH.

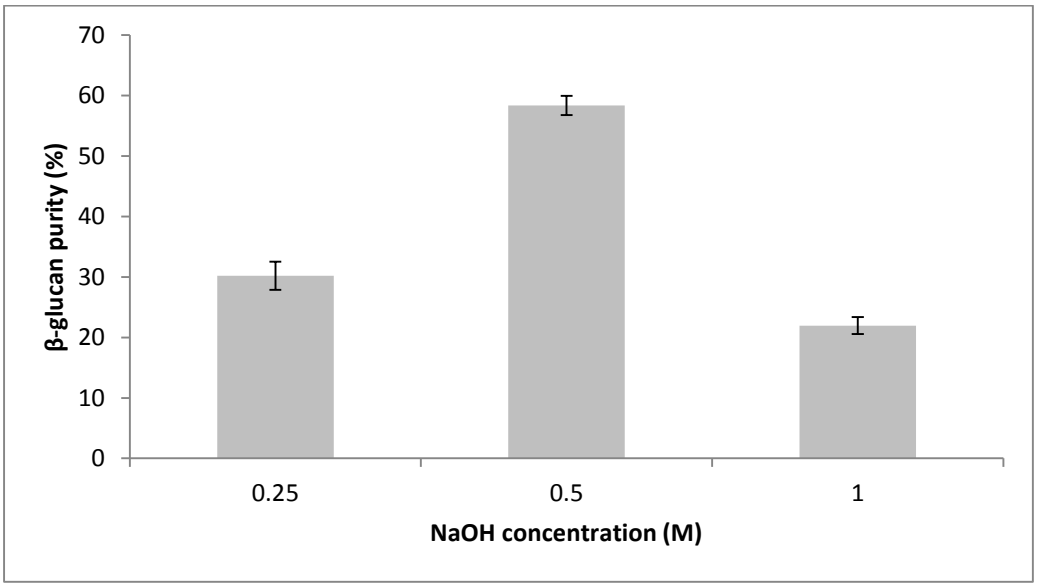

Fig. 2. Preparation of insoluble *β*-glucan from VIN 13 *S. cerevisiae* dry yeast with 10% (w/v) initial cell concentration before autolysis, 24 h autolysis, 20% w/v yeast slurry and NaOH 0.25, 0.5, 1 M. Extraction was performed for 1.5 h at 90^{o}C. Error bars represent standard errors of the average value of all replications with each range of *β*-glucan concentration

3.4.2. Red wine lees by-product

The *β*-glucan extraction from the yeast lees was performed in one step process with the use of hot NaOH solution. Alkali extraction was chosen because it solubilizes all yeast cell wall components but *β*-glucan is received insoluble as sediment [13]. Also, it can be used for treatment of bulk quantities of yeast waste remaining after fermentation in winery tanks. The preparation of *β*-glucan from red yeast lees with NaOH was optimized with four different NaOH concentrations (0.25 M, 0.5 M, 0.75 M, 1 M) in four extraction times (0.5 h, 1.0 h, 1.5 h, 2.0 h) and three different temperatures (80^{o}C, 90^{o}C, 100^{o}C). Also, for the determination of the optimal NaOH extraction conditions, samples of 10 ml from each phase were taken at 0 h, 0.5 h, 1.0 h, 1.5 h and 2.0 h, dried and weight for the determination of yield of *β*-glucan and the loss of the initial yeast waste biomass during the extraction process. The main protocol for the optimization of *β*-glucan extraction was designed for industrial production of *β*-glucan from winery wastes in such way in order to determine the concentration of NaOH needed for the extraction of *β*-glucan, the phase at which the recovered *β*-glucan has obtained the highest purity and the temperature that must be set for the performance of extraction. Also, in order to determine more precisely the optimal extraction conditions, the *β*-glucan concentration in initial yeast lees and after autolysis step was measured. Induced autolysis had a minor effect on *β*-glucan extraction. This is also in agreement with results with the measurement of total polyphenols (data not shown) in this step and from the determination of autolysis ratio. The most purified fraction (19.56 ± 1.28 %) of recovered *β*-glucan was performed with optimal conditions NaOH 0.25 M, temperature 90^{o}C and extraction time 1.5 h. The wine phenolic compounds and the produced alcohol during fermentation inhibit the action of *β*-glucanase used for clarification of must extracted from grapes affected by *Botrytis cinerea* [35]. Due to this fact, it is possible that purity and yield values from red yeast lees treated in the present work for *β*-glucan determination with enzymatic kit containing 1,3-*β*-glucanase, 1,6-*β*-glucanase and chitinase, are higher because of the inhibitive action of absorbed phenols and alcohol from yeast lees on the enzymatic complex. Commercial products available in the market vary in *β*-glucan content from 15-90 % [36]. High purified fractions do not mean best results as the immunostimulant activity of *β*-glucan depends on various parameters (extraction method, degree of brancing, molecular weight, binding with receptors etc.) and crude preparations with high *β*-glucan levels may have adverse

effects for humans. Different *β*-glucans appear different effectiveness and we do not precisely know which structural features are the best for inducing its activity [37]. Differences in *β*-glucan activity are expected even between various *β*-glucans differentially isolated from the same source [38].

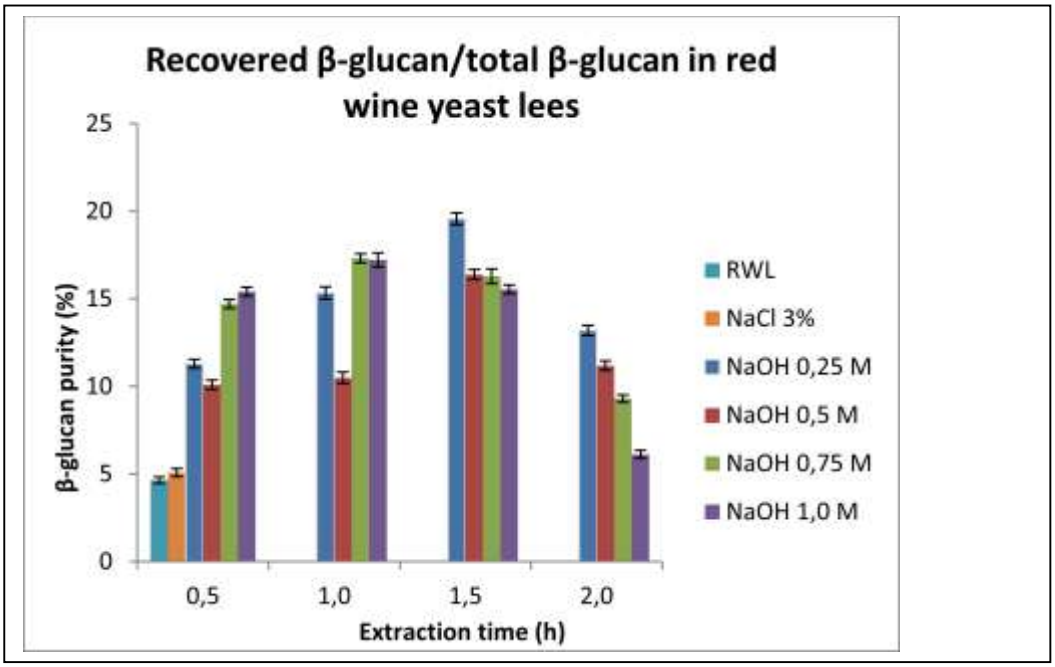

Fig. 3. *β*-Glucan concentration (%) in dry preparations of insoluble *β*-glucan from red yeast lees waste biomass with 20% (w/v) cell concentration and NaOH 0.25 M, 0.5 M, 0.75 M and 1 M at extraction time 0.5 h, 1.0 h, 1.5 h and 2 h. RWL is *β*-glucan content (%) in dry red lees before induced autolysis step. NaCl 3% is *β*-glucan content (%) in dry yeast lees after induced autolysis. Extraction was performed for 2h at 90ºC. Error bars represent standard errors of the average value of all replications with each range of *β*-glucan concentration.

2.3.Extraction of phenolic compounds

During *β*-glucan preparation in red wine lees with alkaline treatments, different fractions of phenolic compounds were recovered. The alkaline phenolics were neutralized with natural acetic acid (see above) (data not shown).

2.4. Loss of dry biomass of yeast lees during extraction process

The freeze-dried *β*-glucan samples taken every 10 min during the extraction process were weighted and used apart for *β*-glucan content determination, also for the loss of initial dry biomass during the extraction process. This was done in order to optimize the yield of the extracted *β*-glucan but also to determine the lost *β*-glucan content released in the supernatant after NaOH application and the obtained polyphenols (data not shown). At the first 0.5 h of the extraction process more than 50 % of the initial biomass was lost as supernatant for all performed NaOH concentrations.

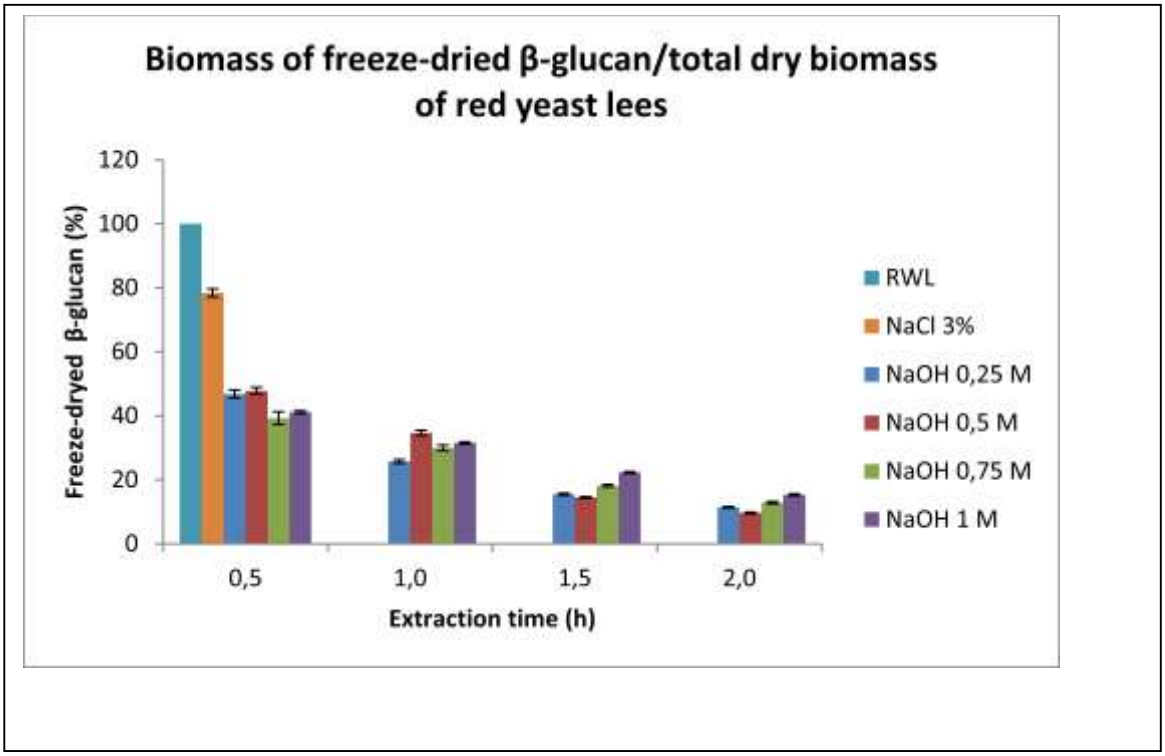

Fig. 4. Dry weight (% of the initial dry biomass) in dry preparations of insoluble *β*-glucan from red yeast lees waste biomass with 20% (w/v) cell concentration and NaOH 0.25 M, 0.5 M, 0.75 M and 1 M at extraction time 0.5 h, 1.0 h, 1.5 h and 2 h. RWL is dry biomass (control 100%) in dry red lees before induced autolysis step. NaCl 3% is dry weight (%) in dry preparation of yeast lees after induced autolysis. Extraction was performed for 2 h at 90ºC. Error bars represent standard errors of the average value of all replications with each range of freeze-dried *β*-glucan concentration.

2.5.Yield

The highest yield 6.34 % ± 1.37 is obtained with NaOH 1 M, extraction time 0.5 h and temperature 90ºC. This value is lower than values reported from Zechner-Krpan et al. (13.64 %) and Araújo et al. (10 %) for *β*-glucan isolated from brewery wastes but wine red lees are structurally more complicated and lower values are expected [14, 28].

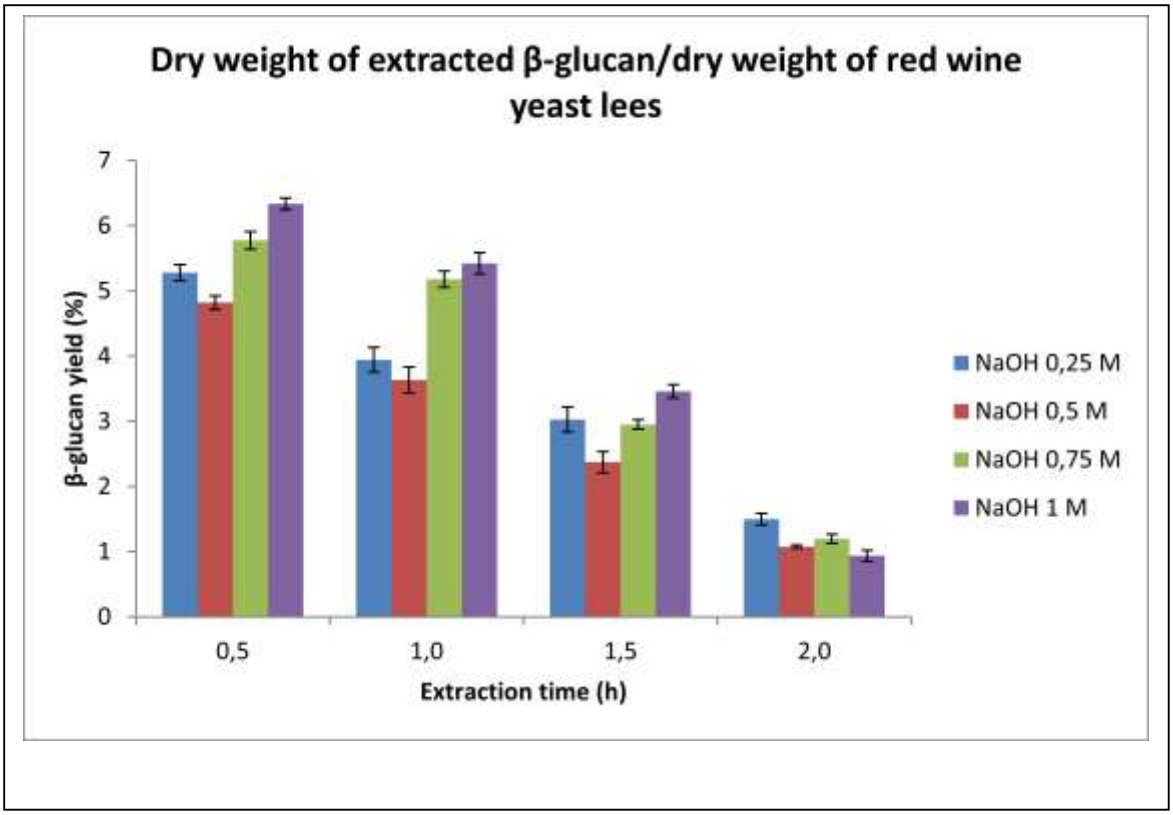

Fig. 5. *β*-Glucan yield (%) in dry preparations of insoluble *β*-glucan from red yeast lees waste biomass with 20% (w/v) cell concentration and NaOH 0.25 M, 0.5 M, 0.75 M and 1 M at extraction time 0.5 h, 1.0 h, 1.5 h and 2 h. Extraction was performed for 2 h at 90ºC. Error bars represent standard errors of the average value of all replications with each range of *β*-glucan yield value.

Conclusions

Yeast waste biomass remaining in the tanks during the wine making process can comprise a new source for *β*-glucan production. Furthermore, the industrial production of added value products, such as *β*-glucans, from winery yeast wastes justifies with the concept of an intergraded, green and environmentally oriented wine industry. Further research on immunotoxicity and immunological activity of recovered *β*-glucans and applications as additives in functional foods and beverages, is proposed. Yeast wine lees, especially red ones, constitute a complex compound which is yet unexplored. We suggest that further research on *β*-glucans with phenolic compounds from red wine lees will offer new perspectives for medical and functional food applications.

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