Bioconversion of Grape Marc into Protein Rich Animal Feed by Microbial Fungi

Florian Zepf and Bo Jin*
School of Chemical Engineering, The University of Adelaide, Australia

Abstract

This investigation was aimed to establish a bioprocess engineering strategy for the bioconversion of grape marc into protein rich animal feed. We experimentally identified influential operation parameters in solid state fermentation (SSF) process, including medium substitutions composition: nutrient, inoculum size and water content, and operation conditions: fermentation time and temperature, and steam treatment time. Three pre-selected microbial fungal strains Aspergillus oryzae DAR 3699, Aspergillus oryzae RIB 40 and Trichoderma reesei RUT C30 were employed in this study. Overall results revealed that these fungal strains performed an excellent bioaccessibility to use grape marc and wine lees as sole carbon nutrient sources. The inoculum size, steam treatment, temperature and water content were found to be the key parameters, which can significantly affect fungal cell growth and protein enrichment. Under optimized conditions, protein content can be increased from 7% to 27% after 5 days of fermentation, resulting in approximately 280% protein improvement. It was expected that the fungi-enriched grape marc can be suitable as highly valuable animal feedstock.

INTRODUCTION

Nowadays, there is a growing interest in the reuse of the organic residues generated by the wine industry, such as vine prunings, grape stalks and gape marc. The grape marc residuals consist of stems, peels and seeds, which are 20% of the grape weight processed into wine industry [1-2]. The grape marc has been used as a carbon source in solid state fermentation (SSF) processes for production of high-value products such as ethanol, citric acid, gluconic acid, carotenoids, xanthan and enzyme production [1,3]. The direct application of grape marc as animal feeds is limited to up to 30% of the feed for ruminants due to its low nutritional value and its antinutritional factors [4].

The solid state fermentation technology has been recognized as a promising biotechnology for the bioconversion of agricultural organic wastes and the biotransformation of crops [5], to produce value-added bioproducts, while minimizing the waste accumulation and the environmental impact. SSF is classified as the cultivation of microorganisms on moist solid substrate in the absence of free water, mimicking the natural environment of the cultivated microorganisms [6]. Although many cellulose-degrading microorganisms, mostly microbial fungi, are known, a few of them are qualified with GRAS (Generally Regarded As Safe) status so that metabolites produced by these strains can be safely used for food-chain products [7]. Aspergillus oryzae and Trichoderma reesei find a wide application in the food, biochemical and pharmaceutical industry for the production of various substances [8-11]. Pariza and Johnson [12] reported examples of fermented foods produced by traditional or modern biological technologies using A. oryzae and T. reesei. The safety of A. oryzae and T. reesei as production organisms for food-grade products has long been recognized, and most industrial production strains have successfully demonstrated a long history of safe use [10,13,14].

Most agro-industrial wastes used by SSF do not contain sufficient bio accessible carbon sources and all the necessary nutrients for microbial growth and metabolic reactions. These organic sources maybe available in sub-optimal concentrations. In these cases, the certain nutrient substrates must be supplemented to stimulate or improve the enzyme production by adding extra carbon sources or nitrogen sources [15]. The nutrient supplementation can also be carried out with the adjustment of the initial moisture content of the residue using a solution containing mineral salts or combining the solid with other residues [16,17]. The objective of the present investigation was to identify key cultivation parameters including medium substitutions composition: nutrient, inoculum size, water content, and operation conditions such as fermentation time and temperature, and steam pre-treatment time. These optimal parameters will be used to develop an SSF engineering strategy.
to improve protein content of the grape marc using recently selected *Aspergillus oryzae* DAR 3699, *Aspergillus oryzae* RIB 40 and *Trichoderma reesei* RUT C30. It was expected that the fungus-enriched grape marc can be suitable as highly valuable animal feedstock.

**MATERIAL AND METHODS**

**Microorganisms**

Three fungal strains *Aspergillus oryzae* DAR 3699 (DAR 3699), *Aspergillus oryzae* RIB 40 (RIB 40) and *Trichoderma reesei* RUT C30 (RUT C30) were selected from our previous investigations and will be used in this study for the optimization of substrate and SSF operation conditions for protein enrichment on the red grape marc. Previous experiments have shown that these selected microbial fungi are suitable candidates for the utilization of grape marc as carbon source in the SSF process. The strains were obtained from CSIRO Food Division, Sydney, Australia and have all graded as food safe (GAFS). The cultures were cultivated on potato dextrose agar slants for 7 days at 30°C, and were maintained in refrigerator at 4°C as seed culture for further use. The seed cultures were re-cultivated bimonthly.

**Vineyard residue**

**Red grape marc**: 1:1.2 The red grape marc (RGM) was provided by the Richmond Grove Winery located in the Barossa Valley in South Australia. The red grape marc was freshly collected after fermentation process for red wine production. The RGM was dried under sunshine for 12 hours and afterwards they were placed in an oven at 50°C for 24 hours until the water content of the grape marc was less than 3%. The grape marc was then stored under refrigeration at 4°C for further use. The dried RGM shows following concentrations in protein content of 7%, digestibility of 30% and residual sugar content of 10% glucose.

**Wine lees**: Wine Lees are a waste product of the winery industry occurs after the fermentation and aging process. The lees consist of dead yeast cells, grape seeds, pulp, stems, skins, and tartrates that get separated from the juice during wine making and aging. The wine lees were collected from Richmond Grove Winery in the Barossa Valley, South Australia. The wine lees was obtained directly from the freshly emptied fermentation lees in combination with the mineral solution to RGM. The fermentation was carried out in shake flask tests using RGM (66.7% water content) and 1 x 10^8 spores g^-1 of the tested fungal strains incubated at 30°C for 7 days.

**Experimental set up**

15g ± 0.25g dried RGM were filled into 250ml Erlenmeyer shake flask and mixed with Milli-Q water obtained from a Barnstead Nanopure ion exchange water system with 18.2M-cm resistivity. The flasks were sterilized at 121°C for 15 minutes, otherwise as stated in the text, and then were cooled down to room temperature. An inoculum size of 1 x 10^8 spores g^-1 substrate, and supplement and minerals as necessary were aseptically added to the prepared grape marc medium, otherwise as stated in the text. The fermentation contents were homogeneously mixed and incubated statically at 30°C for 7 days, otherwise as stated in the text.

The SSF process parameters tested in this study were designed and optimized to achieve the best results of protein enrichment. These important operation parameters included incubation periods (3-14 days), water contents [30-90% (w/w)], incubation temperatures (20 - 35°C), and inoculum size (10^5-10^8 spores g^-1), as well as steam treatment time (15 - 180 minutes). For each experimental variable all other parameters were kept at the same fermentation conditions described above.

**Chemical analysis**: The water content of the fermentation media was determined by drying a fermentation sample at 60°C under vacuum for 8-12 hours to constant mass. For estimation of total sugars, 1g of the substrate was suspended in 50ml of distilled water. The sample was kept at room temperature for 12 hours for extraction of the sugars. The filtrate was then analysed for residual sugars in the form of glucose and fructose using HPLC (Varian Pro-star, Varian Microsorb-MW 100-5 C18 column (4.6 x 150 mm, 5 μm)] at a flowrate of 1.0 mL/min. The biomass was expressed in terms of total protein content. Total protein estimation was done by the Folin method [18]. The digestibility was measured by the standard method in vitro digestibility by Tilley and Terry [19].

**Data analysis**: All results presented in this paper are the means of determinations made on triplicates. The means were determined by using least significant difference values calculated at P<0.05 (Tukey's test).

**RESULTS AND DISCUSSION**

**Inoculum size**

The inoculum size was reported to be an important factor influencing the growth of microbial fungi in a SSF system. The inoculum size was tested in a range of 1 x 10^5 - 10^8 spores g^-1 of *A. oryzae* RIB 40, *A. oryzae* DAR 3699 and for *T. reesei* RUT C30.
Figure 1 shows impact of inoculation size on protein enrichment of red grape marc using three fungi in SSF tests. Poor fungal cell growth was observed in the testing flasks which were inoculated with $1 \times 10^2$ and $1 \times 10^3$ spores g$^{-1}$ substrate. The protein content could only be slightly increased to 10% for the *Aspergillus* sp inoculated tests with inoculum size of $1 \times 10^5$ spores g$^{-1}$ substrate. All three strains performed a promising growth at an inoculum concentration of $1 \times 10^4$ or more spores g$^{-1}$ substrate, resulting in significantly increasing the protein content in the 7 day fermentation. It is worthwhile to note that a significant increase of 21.5% protein content was found in the fermented grape marc using $10^6$ spores g$^{-1}$ of *A. oryzae* DAR 3699. A further increase in the inoculum size over $10^5$ spores g$^{-1}$ resulted in a slight increase in the protein content. However, using *A. oryzae* DAR 3699 and *T. reesei* RUT C30 at inoculum size higher than $1 \times 10^5$ spores g$^{-1}$ substrate the protein content appeared to decline. The best results of protein enrichment can be obtained if the inoculum size is provided in the range of $1 \times 10^5$ - $10^7$ spore g$^{-1}$ substrate for three tested fungal strains.

The inoculum concentration is an influential parameter for any type of fermentation processes regardless SSF or submerged fermentation for the production of microbial metabolites [20]. Therefore, a designed quantity of inoculum for SSF is necessary [21]. This stands in relation with the findings of this research where an inoculum size of $10^5$ spores g$^{-1}$ substrate is needed. Large inoculum sizes are expected to reduce the lag phase and higher production can be achieved in shorter times. The results confirm this that a rapid growth can be achieved with increasing inoculum sizes. However, the protein concentration in RGM could not increase much further between $10^5$ to $10^6$ spores g$^{-1}$ after 7 days of fermentation. This could be due to competition of growth between fungal hyphae over the limited substrate source [22].

**Fermentation time**

The results in Figure 2 present the effect of the fermentation time on the protein contents on grape marc which was inoculated with $1 \times 10^5$ spore g$^{-1}$ substrate for three tested fungal strains, and incubated at 30 °C. One could see that a fermentation time of at least 5 days is required to achieve the best bioconversion performance in terms of fungal cell growth and protein enrichment. The highest protein content could be achieved as high as 20.2% and 17.8% by *A. oryzae* RIB 40 and *T. reesei* RUT C30, respectively, after 5 day fermentation. However, the protein contents appeared to be declined slightly as 18.4% and 16.5%, respectively, after 7 day fermentation. The highest protein content of 20.4% was obtained in the fermentation test using *A. oryzae* DAR 3699 after 6 days, while the protein content decreased gradually and reached 19.7% after 14 day fermentation. It was found that a longer fermentation time could only result a slight increase in the protein content. The results suggest that a fermentation time of 5 days is recommended for the protein enrichment of red grape marc residual.

To achieve a high protein enrichment of grape marc residual, our results revealed that at least 5 days of cultivation are necessary. A prolonged fermentation time could not be beneficial to improve protein concentrations, if the fermentation rate is taken into a consideration. After 14 days of cultivation with *T. reesei* RUT C30 the protein concentrations even declined. This might be due to denaturation and/or decomposition as a result of interactions with other compounds in the fermented medium or dying of mycelia. Five days of solid state fermentation of grape marc was recommended for protein enrichment for the tested microorganisms.

**Fermentation temperature**

Figure 3 presents the results of protein contents in the SSF with respect to the growth temperature, which was tested between 20°C and 35°C. The experimental data revealed that all three fungal strains performed a sensitive growth status in the testing temperature rage. The protein contents increased exponentially as the growth temperature increased from 20°C to 27°C.

The optimum temperature for protein enrichment after 5 days of fermentation was found to be within the range of 27°C - 30°C, leading to the maximum protein contents of 21.2%, 23.2% and 17.8% for *A. oryzae* RIB 40, *A. oryzae* DAR 3699 and *T. reesei* RUT C30, respectively. If the fermentation temperature rises above 30°C the protein content declined considerably. The highest protein content was achieved at 28°C, which was selected as the optimal operation temperature for the SSF process using the three testing fungal strains.

Effect of incubation temperature is rather an organism-dependent parameter. The growth temperature is an essential factor which affects the microbial SSF performance [23,24]. Furthermore the accumulation of heat, which is directly proportional to the metabolic activity of the microorganism, can
raise the temperature in the fermentation medium during the fermentation period, resulting in a possible temperature increase up to 20°C [25]. If the fermentation temperature is higher or lower than the optimum range the cellular activities might be inactive or enzymatic reactions could be terminated. This explains the results of fungal cell growth and protein enrichment under and above the optimum fermentation range for the microorganisms tested.

Water content

Figure 4 illustrates the effect of the water content on fungal cell growth, which is presented as protein production in the grape marc with controlled water contents. The grape marc with a water content higher than 80% results in releasing free water, which is not suitable for fungal growth. Poor fungal cell growth was found in the grape marc which had water content below 30%. Therefore, the designed water contents were controlled between 30% and 75% in this study. In general, the protein content increased significantly with the water content varied between 30% and 75% in this study. For the SSF process the substrate water content is an important factor which influences directly microbial growth and metabolic production. The water contents above the optimum level lead to a reduced porosity of the solid medium, resulting in a lower oxygen transfer rate. The water contents below the optimum level result in the reduction of nutrient solubility, and lower degree of swelling [14,26]. In the case of SSF of grape marc the water contents higher than 75% could not be tested because of the water-holding capacity. The tested fungi prefer a moisture range of 55% to 67%. Thus, the water content lower and higher than this range is not favorable for the fungal cell growth. In some cases, the moisture level in SSF system may increase due to the production of metabolic water [27].

Steam treatment time

The steam treatment of grape marc can be a promising step for the success in operation of the SSF for protein enrichment of the grape marc. This is necessary to minimize possible contamination by foreign microorganisms existed in the grape marc. Our preliminary trials revealed that all fermentation trials using the raw or fresh grape marc without steam treatment failed due to contamination and growth of unwanted foreign microorganisms. Furthermore, this steam treatment is able to enhance biochemical accessibility of the grape marc, which can be beneficial for the fungal cell growth, and thus promotes the SSF performance and protein enrichment rate. The steam treatment times were set up from 15 up to 180 minutes. The impact of the steam treatment time on the fungal cell growth is presented in Figure 5. The results show that the steam treatment between 15 minutes and 60 minutes showed positive effects on the protein production. The protein content for all three strains can be enhanced significantly. The best results could be achieved for A. oryzae RIB 40 where protein concentrations were 20% after 15 minutes and 27.2% after 60 minutes. A prolonging steam treatment over 60 minutes show a little improvement in the protein concentration for the fermentation trials using A. oryzae RIB 40 and A. oryzae DAR 3699, while a slight increase in protein content was found in the test using T. reesei RUT C30. Therefore, in consideration of production efficiency and energy costs associated with steam treatment and cooling, 60 minutes can be seen as the optimum steam treatment time for the protein enrichment of grape marc in the SSF.

Our results revealed that the steam treatment is promising to not only minimize contamination, but also to enhance the bioaccessibility of the grape marc, resulting in improving the fungal cell growth and fermentation efficiency. It is worthwhile to note that water content of the grape marc can be maintained at approximately 50-60% after the steam treatment, which is a suitable water content level for fungal cell growth. Hu et al. [28] reported positive effects of the sterilization of pangola grass for the protein enrichment in comparison to non-sterilized substrate. The sterilization process through the steam treatment enhances the bioconversion of the substrate, stimulates microbial growth and improves the degradation of solid substrates. The most economic sterilization time for protein enrichment of grape marc was determined as 60 min in this study.
Nitrogen sources

Previous studies revealed that the supplementation of nitrogen sources with trace elements showed better results in the SSF than these with a nitrogen source only [5,15]. Herein, we tested nitrogen sources in combination with trace elements. The composition of the trace element solution can be seen under section 2.4. Experiments were carried out using four commonly used nutrient sources: yeast extract, ammonium sulphate, bacterial peptone and soy peptone at different concentrations. The effects of various organic and inorganic nitrogen sources on the protein enrichment in RGM are indicated by the results given in Figure 6.

The SSF results presented in Figure 6 show that the protein production can be increased with the supplementation of the nitrogen sources in combination with trace elements. In comparison with experimental data shown in Figures 1-5, the trace elements, as these without addition of nitrogen sources, performed a limited improvement for fungal cell growth and protein enrichment. Overall results show that in terms of nutrient accessibility A. oryzae DAR 3699 was the best producer followed by A. oryzae RIB 40 and T. reesei RUT C 30. Ammonium sulphate was found to be the best nitrogen source for protein enrichment, followed by yeast extract. On the other hand, bacterial peptone and soy peptone appeared to be less favoured nitrogen sources for the fungal growth. A high protein content was found corresponding to a nutrient supplementation range of 0.6% to 1%. A supplement concentration of 0.6% for any nitrogen sources seems to be the optimum for the tested fungal strains in the SSF. The results show that the maximum protein enrichment of 26.8% was produced by A. oryzae DAR 3699 at a concentration of 1% for ammonium sulphate at, followed by yeast extract 26% and bacterial peptone 25.6%. As light lower yield of protein enrichment was observed with soy peptone 24.6%. Nevertheless, the supplementation of the nitrogen sources in combination with trace elements could lead to an increase in protein content from approximately 17-20% up to a maximum level of 26%.

An organic material that provides all the nutrients necessary for the growth of the microorganisms should be considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement them externally [29]. Therefore, the nutrient supplementation is an important approach to enhance the microbial growth mainly during the initial stage of the fermentation which initiates the production of degrading enzymes which can be achieved by supplementation. Our findings showed that supplemented nitrogen sources without minerals might have limited improvement. The fermentation productivity can be significantly increased when the grape marc was added with nitrogen sources in combination with minerals. The use of a nitrogen source, such as ammonium sulphate and yeast extract, at above 1% lead to a reduction in protein content. This finding is in correlation with the findings of Naraian et al. [30], who reported that higher urea and ammonium sulphate concentrations of 1% and 1.5% lead to a reduced growth rate of fungal mycelium compared to 0.5%.

Wine Lees as an alternative nutrient source

Wine lees is another waste product in winery process. The wine lees refers to deposits of dead yeast or residual yeast and other particles that precipitate in the wine fermentation tank. The lees may contain sufficient nutrient sources for SSF process for the RGM bioconversion. The supplementation of wine lees for the fungi-simulated SSF process showed promising results in improving the protein contents using three tested fungi. The data presented in Figure 7 show that the protein concentration with supplementation of wine lees can be significantly increased.
A supplementation with 10% (w/w) wine lees to the grape marc appeared to have the best results in terms of protein enrichment. A slight improvement of fungal biomass growth was given if wine lees was provided at a concentration higher than 15% (w/w). However, the highest protein concentrations could be achieved with supplementation of 25% (w/w) wine lees to the grape marc residual with 26.8% protein for A. oryzae DAR 3699. These results of protein enrichments are comparable with those fermentation tests with supplementation of nitrogen sources, such ammonium sulphate and yeast extract, as shown in Figure 6. By 10% wine lees supplementation the protein concentrations achieved to 26% for two A. oryzae strains and 24% for T. reesei RUT C30. In comparison to the grape marc without wine lees added, supplementation of wine lees resulted in increasing protein content by 18% for A. oryzae DAR 3699 and 30% for A. oryzae RIB 40, while the highest increase of 41% was tested in the grape marc fermented by T. reesei RUT C30.

It is interesting to note that the fermentation test using the wine lees as nutrient source demonstrated comparable results in terms of the fungal cell growth and the protein enrichment with those nutrient supplied SSF using yeast extract, ammonium sulphate, bacterial peptone and soy peptone. This finding indicated that the red grape marc and wine lees from the wine production can provide sufficient carbon and nutrient sources for the bioconversion process using the selected microbial fungi.

CONCLUSION

In summary, we have experimentally determined the optimal substrate composition and operation parameters, including inoculum size: 1 x 10^7/g substrate, fermentation time: 5 days, fermentation temperature: 28°C-30°C, water content: 60-66.7%, steam treatment: 60 minutes, (NH_4)_2SO_4 supplement: 0.60%, and wine lees supplement: 25%. The key parameters, which can significantly affect fungal cell growth and protein enrichment, are the inoculum size, steam treatment, temperature and water content. The selected three fungal strains of Aspergillus oryzae DAR 3699, Aspergillus oryzae RIB 40 and Trichoderma reesei RUT C30 demonstrated promising biochemical associability to use grape marc as a carbon and nutrient source. Under the optimum operation conditions as determined in this study, the protein concentration could be increased from original content of 7% up to 27%, resulting in a significant increment of more than 280% total protein contents for the red grape marc after 5 days of fermentation.

The microbial fungi-induced SSF for protein enrichment of low-grade wastes like RGM for animal feed is a sustainable biotechnological approach. Our results revealed that the red grape marc and wine lees from the wine production can provide sufficient carbon and nutrient sources for the bioconversion process using the selected microbial fungi. The growth studies of the three fungi A. oryzae DAR 3699, A. oryzae RIB 40 and T. reesei RUT C30 using red grape marc have provided a possibility for protein enrichment of vineyard residual for the purpose of animal feedstock production in larger scale. Our experimental results and determined operation parameters could assist to establish bioprocessing engineering strategy of SSF process.

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REFERENCES


Figure 7 Protein concentration of red grape marc supplemented with wine lees in the SSF using T. reesei RUT C30, A. oryzae DAR 3699 and A. oryzae RIB 40.


