Production of Bio-Fungicide from Sugarcane Bagasse using *Pichia membranifaciens* Yeast and its Activity Against Post-Harvest Pathogenic Fungi

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Received: 30.09.2020; Revised: 22.10.2020; Accepted: 23.10.2020; Published: 26.10.2020

Abstract: Antagonistic yeast *Pichia membranifaciens* can produce killer toxins and hydrolytic enzymes to degrade pathogenic fungi cell walls. In this study, sugarcane bagasse was hydrolyzed and used as a low-cost carbon source to produce bio-fungicide using *P. membranifaciens*. In a growth medium obtained from bagasse hydrolysis containing 25 g/l sugar, with optimized amounts of nitrogen sources (2% (w/v)), NaCl and K$_2$HPO$_4$ (0.5% (w/v)) and in the presence of hydrophilic surfactant, Triton X100 (0.02% (w/v)), the yeast was able to produce 3782 mg/l bio-fungicide. The activity and toxicity of the produced bio-fungicide were tested against some post-harvest pathogenic fungi, including *Aspergillus niger*, *Penicillium digitatum*, and *Phytophthora capsici*. Minimum biocidal concentration (MBC) and minimum inhibitory concentration (MIC) were determined to be 378.2 and 37.82 mg/l, respectively. According to the results, the produced bio-fungicide has the potential for preserving agricultural products in the warehouse.

Keywords: antagonistic yeast; *Pichia membranifaciens*; post-harvest pathogens; *Aspergillus niger*; bio-fungicide.

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1. Introduction

Filamentous fungi that produce mycotoxin can cause plant diseases, reduce the quality and yield of crops, and in addition to economic damage, pose risks for human and animal health. Although the estimation of post-harvest mortality, particularly through fungal diseases, is not precise, some data indicate that, on average, about 30% of crops destroy between production and consumption [1]. Post-harvest diseases might be controlled through the treatment of fruits and crops with synthetic chemical fungicides [2]. However, with the demand for pesticide-free fruits and vegetables, and according to the new regulations, the use of chemical pesticides should be reduced [3].

Biological control of agricultural pests by antagonistic yeasts has attracted considerable attention as a promising alternative for chemical pesticides with less environmental impact, which can be used alone or as part of pest management practices to reduce the use of synthetic fungicides [4]. Antagonistic yeasts are of significant importance for pathologists due to their ability to produce extracellular secondary metabolites [5]. These secondary metabolites that are made up of protein adversely affect pathogenic fungi and can kill them. The toxicity of
these proteins depends on their ability to enter the fungi cells [6]. Several antagonistic yeasts can produce such toxins, called killer phenotypes, among them are *Pichia sp.* [7, 8].

In 1992, Marquin *et al.* [9] found that *P. membranifaciens*, which was isolated from olive brine, had a wide range of killer toxin activity. It can produce two types of toxin (PMKT and PMKT2 [10]), which affect the cell wall of pathogenic fungi. PMKT has an affinity to (1, 6) -d-β-glucan, while PMKT2 is absorbed by cell wall proteins [11]. Later, Masih *et al.* [12] simultaneously cultivated *P. membranifaciens* and pathogenic fungus *Botrytis cinerea* on a plate, where *P. membranifaciens* could suppress the grey mold disease by destroying the cell wall of *B. cinerea* and causing its cytoplasm leakage. Santos *et al.* [13] isolated 42 species of *Pichia sp.* from 20 different sources and tested them against 18 strains of pathogenic *B. cinerea*. Results showed that *P. membranifaciens* and *P. anamola* showed the highest inhibition against *B. cinerea*.

In addition to the production of killer toxin against pathogenic fungi, *P. membranifaciens* produces hydrolyzing enzymes including exochitinase, endochitinase, and β-1,3-glucanase [14], and antifungal volatile organic compounds such as 2-nonanone, 2-phenethyl alcohol, 1,3,5,7-cyclooctatetraene and 3-methyl-1-butanol, which cause the death of pathogenic fungi [15, 16]. Cao *et al.* investigated the synergistic effects of *P. membranifaciens* and other controllers such as CaCl$_2$ [17] and methyl-jasmonate [18] to control *Colletotrichum acutatum*, and ammonium molybdate [19], and BTH (benzothiadiazole-7-carboxylic acid-s-methyl ester) [20] to control *Penicillium expansum*. Their results showed that simultaneous treatment led to better control of post-harvest decay due to the increased activity of the enzymes. Zhang *et al.* [21] assessed the inhibition of *P. membranifaciens* against *Monilinia fructicola*, and a 76% reduction in the decaying rate was shown *in vivo*. Since *P. membranifaciens* is usually used as an antagonist agent for control of post-harvest pathogens during the cold storage of fruits [22], for practical purposes, it is important to develop formulations of it which could be easily used at a large scale. In this regard, Zhang *et al.* [23] compared the biocontrol efficacy of vacuum-dried and fresh *P. membranifaciens* on citrus fruit and demonstrated that no significant difference in the biocontrol effects of fresh and active dried yeast was observed.

Despite all efforts, problems such as high production costs hinder the commercialization of bio-fungicides [24]. With the aim of reducing the production cost of bio-fungicides, sugarcane bagasse was used as an inexpensive substrate in this study to produce bio-fungicide using *P. membranifaciens*. The killer activity of the produced bio-fungicide against some post-harvest pathogenic fungi, including *P. digitatum*, *A. niger*, and *P. capsici* was investigated.

### 2. Materials and Methods

#### 2.1. Yeast and pathogenic fungi.

The yeast *Pichia membranifaciens* IBRC-M 30146 was purchased from the National Iranian Biological Resource Center (IBRC), then cultivated in a standard YMB medium containing 0.3% (w/v) yeast extract (Himedia, Iran), 0.3% (w/v) malt extract (Merck, Germany), 0.5% (w/v) peptone (Himedia, Iran) and 1% (w/v) glucose (Merck, Germany) at 25 °C and pH 6.8.

Pathogenic fungi on which the killer activity of the produced bio-fungicide was tested were provided from Partonar Company, Alborz, Iran. For this purpose *Aspergillus niger* causes
black mold disease of grains and fruits, *Penicillium digitatum* causing green mold disease of citrus [25], and *Phytophthora capsici* that causes mold disease of Cucurbitaceae family were used.

2.2. Sugarcane bagasse.

Sugarcane bagasse was provided from a local market, Mazandaran, Iran. It was crushed, dried, and then characterized to determine its total sugar, glucose, and protein content. The bagasse was then hydrolyzed by dilute H₂SO₄ (0.25% v/v) [26], and the obtained hydrolysate was used as a sugar source for the cultivation of the yeast. The total sugar content was determined by the phenol sulfuric acid method [27], the glucose content of the hydrolysate was determined by the dinitrosalicylic acid (DNS) method [28], and the amount of protein was estimated by the Bradford method [29] using standard bovine serum albumin (EquiTech-Bio, America).

2.3. Medium optimization.

In addition to a carbon source, microorganisms require different nutrients to grow and reproduce. In this regard, the effects of various nitrogen sources (urea, yeast extract, malt extract, peptone, ammonium chloride (NH₄Cl) and ammonium sulfate ((NH₄)₂SO₄)), mineral salts (NaCl, CaCl₂, MgCl₂, MgSO₄, FeSO₄, KH₂PO₄, and K₂HPO₄) and hydrophilic surfactants (Triton X-100 and Tween 80) on product formation were investigated, and if they had a positive efficacy on production, their optimal value was determined using the one-factor-at-a-time approach. Since protein (bio-fungicide) production is mostly affected by nitrogen source, at first, different nitrogen sources (1% (w/v)) were tested in the medium obtained from bagasse hydrolysis. Afterward, the nitrogen source with the highest effect on bio-fungicide production was selected, and its concentration was optimized in the range of 1 to 9% (w/v). In the next step, the influence of the surfactant on the protein production was studied in the range of 0.01 to 0.04% (w/v), and the optimum amount of the preferred surfactant was determined. Then, the impact of mineral salts (0.1 (w/v)) on the bio-fungicide production was investigated, and the optimum concentration of the selected salts was determined.

2.4. Bio-fungicide production.

*P. membranifaciens* (3% v/v) were inoculated into a hydrolysate medium containing nitrogen source, mineral salts, and surfactant and incubated at 25 °C and pH 6.8. In the course of fermentation, cell dry weight (CDW), sugar consumption, and protein production were monitored. Bio-fungicide is an extracellular protein product that cells secrete it into the liquid medium. To measure the protein content, an aliquot of the culture was centrifuged at 6000 rpm at 4 °C for 10 min. Then, under a sterile condition, the supernatant was passed through a 0.45 µm syringe filter, and its protein content was measured following the Bradford method.

2.5. Killer activity assay.

 Toxicity and activity of the bio-fungicide were investigated by disk diffusion and direct contact test. After separating the cells by centrifugation and filtering, the remaining filtrate, which contained bio-fungicide, was tested for killer activity.

To observe the effect of the bio-fungicide on the pathogenic fungi and their hyphae, pathogenic fungi were inoculated in 50 ml media in two groups, one as control and the other
as a test group, then incubated at 25 °C for 24 h. Afterward, 30 ml of the filtered bio-fungicide was added to the test group and incubated for another 24 h. Next, the difference between the two groups was examined visually.

2.5.1. Diffusion test.

YMA medium was prepared in several Petri dishes. Then a 200 μl of each pathogenic fungi suspension, which was grown on the YMB medium overnight, was inoculated on the Petri plates and spread uniformly over the agar medium. Then several wells with a diameter of 10 mm were created on the agar surface, and in each well, 100 μl of filtrate with different concentrations of bio-fungicide was added. The plates were then incubated at 25 °C for 24 h. The created halos around each well was an indication of the activity and toxicity of the bio-fungicide [30].

2.5.2. MIC and MBC assays.

The fungicidal efficacy of the bio-fungicide was examined using the standard broth dilution method (CLSI-M07-A9-2012). The bio-fungicide was placed in direct contact with the pathogenic fungus in a liquid medium. The method was used to determine and measure the minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC), through the macro dilution method. The MIC and MBC were determined in YMB using serial dilutions of bio-fungicide in concentrations ranging from 3782 to 3782×10⁻⁸ mg/l with adjusted fungus concentration 0.10 at 625 nm (1×10⁸ CFU/ml, 0.5 McFarland’s standard) [31, 32]. The negative control used in this study contained YMB with tested fungus, and the positive control included YMB and the filtrate containing the bio-fungicide (1:1). The temperature and time of incubation were 25 °C and 24 h, respectively. After 24 to 48 h the liquid was cloudy in some test tubes. The MIC and MBC were noted by the turbidity of the test tubes before and after incubation. The growth ability of pathogenic fungi in each tube in the presence of different concentrations of the bio-fungicide was measured by optical density reading at 600 nm using a spectrophotometer. To check the toxin effect, the culture was centrifuged, and the residual sediment was washed several times with physiological serum, and then grown on YMA medium, and the growth of colonies was investigated. All tests were carried out in triplicate to ensure the accuracy of the results.

3. Results and Discussion

3.1. Substrates and nutrients.

Characterization of sugarcane bagasse indicated that it contained 75.3% total sugar and 7.1% protein. The sugarcane bagasse was subjected to dilute acid hydrolysis, and then the hydrolysate with glucose content of around 25 g/l was used to grow the P. membranifaciens. Figure 1 shows the CDW, sugar consumption, and bio-fungicide (protein) production within 120 h fermentation. As can be seen in the figure, the medium obtained from the bagasse hydrolysis initially contained some protein (1095 mg/l) that was consumed by the microorganism, and its concentration dropped drastically within 18 h of fermentation. Then, the production of protein as the product commenced along with cell mass production and continued up to 86 h when the product concentration reached its maximum concentration of
1075 mg/l. Thereafter, the microorganism began to consume the product due to the expiration of the sugar content in the culture, which resulted in a decrease in protein concentration.

Figure 1. CDW, sugar consumption, and protein production by *P. membranifaciens*.

The production of bio-fungicide in the bagasse hydrolysate medium in the absence and presence of various nitrogen sources was investigated; the results are presented in Figure 2 A. Results showed that the cell metabolic pathway was strongly influenced by the nitrogen source. Urea and ammonium chloride caused severe alkalinity in the medium and needed to be neutralized with acid first. These nitrogen sources did not show any encouraging effect on bio-fungicide production; even in the presence of urea, the protein production reduced compared to the nitrogen-free medium. In the case of the other nitrogen sources, the addition of 1% (w/v) of each nitrogen source to the bagasse hydrolysate was effective in enhancing the protein production compared to the control medium. The combination of three nitrogen sources, i.e., peptone, yeast extract, and malt extract (PYM), with the mass ratio (5:3:3), had the highest effect on the bio-fungicide production. In the presence of PYM as a nitrogen source (1% (w/v)), 2215 mg/l protein was synthesized by the cells, which was higher than that produced by individual nitrogen sources. Hence, the content of PYM in the medium was varied in the range of 1 to 9% (w/v), and its effect on protein production was monitored (Figure 2 B). The increase of the PYM from 1 to 2% (w/v) considerably improved the concentration of the synthesized bio-fungicide from 2215 to 2900 mg/l; however, further increase of the nitrogen source concentration in the medium was not effective to enhance the product formation.

The effect of two hydrophilic surfactants, Tween 80 and Triton X100, on product formation was investigated, as shown in Figure 2 C. Triton X100 was more effective than Tween 80 at the same concentration, and optimum surfactant concentration was determined to be 0.02% (w/v). At this concentration of surfactants, the product formation was 3222 and 3429 mg/l in the presence of Tween 80 and Triton X100, respectively. Surfactant helps to dissolve more oxygen in the liquid phase and hence provides better accessibility to oxygen for the aerobic yeast. Ions and salts also affect the metabolic activity of the cell; they act as the original regulator of the production enzymes and/or stimulants in the growth medium. On the other hand, some salts, especially at high concentrations, have inhibitory effects on the yeast. In this regard, the presence of several salts in the growth medium was individually examined to select the salts and ions that have a positive effect on bio-fungicide production. Figure 2 D shows the effect of different salts and ions on bio-fungicide production. The medium containing NaCl
and K₂HPO₄ revealed increased protein production; however, other minerals and salts such as FeSO₄, MgCl₂, MgSO₄, KH₂PO₄, and CaCl₂ had no or even negative effect on product formation. The optimal amounts of NaCl and K₂HPO₄ were determined as 0.5% (w/v) with product formation of 3518 and 3782 mg/l, as exhibited in Figure 2 E and F, respectively.

![Figure 2](https://biointerfaceresearch.com/)

**Figure 2.** Optimization of nutrient sources for the bio-fungicide production: A) choice of nitrogen source, B) optimization of nitrogen source concentration, C) effect of surfactant and its concentration, D) effect of different salts, E) effect of NaCl concentration, and F) effect of K₂HPO₄ concentration.

3.2. Killer toxin activity.

3.2.1. Diffusion test.

The effect of bio-fungicide on the growth of pathogenic fungi is shown in Figure 3. After 24 h, the growth of pathogenic microorganisms around the wells at the surface of the plate was prevented. On the plate containing *P. digitatum*, there was no trace of growth at high concentration, and the plate was completely clear. This indicates that the produced bio-fungicide had a greater killing effect on *P. digitatum* compared to other fungi.

The diameter of the halo around each well was measured, as shown in Table 1. The increase in the concentration of the bio-fungicide increased the diameter of the inhibition area. *P. digitatum* revealed a larger inhibition zone than other pathogenic fungi, and it was more affected by the bio-fungicide.

| Label | Halo diameters (mm) | Label | Halo diameters (mm) | Label | Halo diameters (mm) |
|-------|---------------------|-------|---------------------|-------|---------------------|
| A     | 0                   | E     | 0                   | I     | 0                   |
| B     | 3.9 - 12.9          | F     | 1.3 - 3.9           | J     | 0 - 9.0             |
| C     | 12.9 - 38.7         | G     | 9.0 - 14.2          | K     | 6.5 - 12.9          |
| D     | 38.7                | H     | 9.0 - 21.9          | L     | 9.0 - 15.5          |

3.2.2. MIC and MBC determinations.

As shown in Figure 4, in the test tube with the bio-fungicide concentration of 3782 ×10⁻¹ mg/l, no fungi growth was observed, and in the next tube, with a bio-fungicide concentration of 3782 ×10⁻² mg/l, the growth was inhibited. Accordingly, the concentrations of 3782 ×10⁻¹ and 3782 ×10⁻² mg/l were determined as MBC and MIC, respectively.
Figure 3. The inhibitory effect of the produced bio-fungicide at different concentrations on pathogenic fungi: (A-D) *P. digitatum*, (E-H) *A. niger*, and (I-L) *P. capsici*.

Figure 4. Determination of MIC and MBC for pathogenic fungi.
Measuring the fungi cell growth in the liquid culture and their opacity was carried out to assess the killer activity of the produced bio-pesticide; the results are illustrated in Figure 5. Results indicated that the produced bio-fungicide was most effective on the growth of pathogenic fungus *P. digitatum*, followed by *A. niger* and *P. capsici*. In the tubes with the same concentrations of the bio-fungicide, the growth of *A. niger* and *P. capsici* was higher than *P. digitatum*, indicating that the pathogenic fungus *P. digitatum* was more susceptible to the bio-fungicide and its growth was more hindered in the presence of the toxin.

![Figure 5](https://doi.org/10.33263/BRIAC113.1043510445)

**Figure 5.** The growth ability of pathogenic fungi in cultures with different concentrations of bio-fungicide.

In the present study, when pathogenic fungi were grown on the YMB, and the bio-fungicide was added, the fungi failed to germinate, and their hyphae were destroyed, as depicted in Figure 6. As observed, in the bottle which contained the bio-fungicide, no growth of pathogenic fungi was observed, signifying the killer activity of the bio-fungicide. Table 2 presents a summary of the reports available in the literature on the inhibitory effect of *P. membranifaciens* on various pathogenic fungi. The focus of the current study was on the utilization of a low-cost substrate to reduce the production cost of bio-fungicide. Results of the current study indicated that the use of bagasse hydrolysate as the growth medium and optimization of the nutrients and element added to the medium resulted in the successful production of bio-fungicide, which could effectively inhibit the growth of some pathogenic fungi.

![Figure 6](https://biointerfaceresearch.com/)

**Figure 6.** A-C) Control group: Growth of pathogenic fungi on YMB and D-F) Test group: destroyed hyphae of pathogenic fungi in the presence of 3782 mg/l of bio-fungicide in YMB.
Table 2. Summary of studies on the inhibitory effect of *P. membranifaciens* on pathogenic fungi.

| Pathogenic microorganism | Disease            | Media | Remarks                                                                 | Ref.  |
|-------------------------|--------------------|-------|-------------------------------------------------------------------------|-------|
| *Botrytis cinerea*      | Grey mold          | YNB   | Coagulation and leakage of the cytoplasm of pathogenic fungus          | [12]  |
| 18 strains of *B. cinerea* | Grey mold          | YMB   | *P. membranifaciens* had an inhibitory effect via killer toxin activity | [13]  |
| *Penicillium roqueforti* |                   | MEB   | *Pichia* strains strongly inhibited *P. roqueforti* in the mini-silos    | [33]  |
| *Colletotrichum acutatum* | Anthracnose rot   | NYDB  | Improved biocontrol and increased enzyme activity in the presence of CaCl$_2$, inhibited spore germination of fungus | [17]  |
| *Colletotrichum acutatum* | Anthracnose rot   | NYDB  | Methyl jasmonate remarkably improved control and increased the population of antagonist yeast against pathogen fungus | [18]  |
| *Brettanomyces bruxellensis* |                  | YMB   | Biocontrol activity of PMKT2 in winemaking condition, preventing contamination | [34]  |
| *Penicillium expansum* | Blue mold          | NYDB  | Ammonium molybdate increased the growth of *P. expansum* and improved the efficacy control of disease | [19]  |
| *Penicillium expansum* | Blue mold          | NYDB  | Hydrolytic enzymes synergistic effect with benzo-thiadiazole-7-carbothioic acid S-methyl ester (BTH) | [20]  |
| *Penicillium expansum* | Blue mold          | NYDB  | *Lentinula edodes* improved the efficacy, induced higher phenolic accumulation, and up-regulation of enzyme activity | [35]  |
| *Monilinia fructicola*  | Brown rot          | NYDB  | Controlled 76% of disease in vivo                                      | [21]  |
| *Colletotrichum gloeosporioides* |                | PDB   | Reduced fungal growth, competition for limited resources and antibiotics | [36]  |
| *Penicillium expansum*  | Blue and grey mold | Pear juice | Adding several amino acids and/or CaCl$_2$ resulted in improved antagonistic activity | [37]  |
| *Fusarium spp and Verticillium spp* | Coffee rust | Coffee | Slowing down the progress of the rust disease                           | [38]  |
| *Fusarium verticillioides*  | Sweet corn seed   |       | Reduced fumonisin production by *F. verticillioides* by 73% and reduced fumonisins by *F. proliferatum* by 56% | [39]  |
| *Pinicillium digitatum*  | Green mold         |       | Production of bio-fungicide inhibited the growth and destroyed hyphae of pathogen fungi, and at 3782 mg/l concentration could annihilate the pathogenic fungi | [This study] |
| *Phythophtora capsici*   | White mold         | Bagasse hydrolysate |                                                  |       |
| *Aspergillus niger*      | Black mold         |       |                                                                      |       |

4. Conclusions

In this study, the production of bio-fungicide from sugarcane bagasse as a cost-effective carbon source using the yeast *P. membranifaciens* was investigated. The activity and toxicity of the produced bio-fungicide on three pathogenic fungi, *P. digitatum*, *P. capsici*, and *A. niger* known as fruit rotting pathogenic fungi, were demonstrated. The results of this study were promising in the sense that lignocellulosic wastes can be used as a low-cost carbon source for the production of bio-fungicide for preserving agricultural crops and products against post-harvest pathogenic fungi.

Funding

This work was supported by Biotechnology Research Laboratory, the Babol Noshirvani University of Technology through MSc grant no. BNUT/935150006/97.

Acknowledgments

The author also would like to thank the research and development group of Partonar Company for their technical assistance in pathogenic fungi studies.
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