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Research Article

Biomass production and formulation of biocontrol yeast *Candida membranifaciens*

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Abstract: In this study, the effect of carbon and nitrogen sources, on production of yeast biomass was determined as well as the efficacy of various formulations of *Candida membranifaciens* to decrease blue mold on apple. The media containing sugar cane molasses as carbon sources caused more yeast growth than the media supplemented with permeate powder. Yeast extract as nitrogen source produced higher biomass than urea and Corn steep liquor (CSL). The best growth was obtained in medium that composed of sugar cane molasses, yeast extract and CSL. Viability of *C. membranifaciens* differed in various carriers significantly ($p < 0.01$). The number of viable cells in Kaolin and Talc-based formulations was gradually reduced whereas the viability in wheat bran based formulations increased in 4 months and then gradually declined. In general, the formulations stored at 4 °C had longer shelf life than those stored at 24 °C. The antagonistic efficacy of prepared formulations of *C. membranifaciens* was evaluated against *Penicillium expansum* on apple at storage conditions. There were no significant differences among Talc-based, Kaolin-based and Wheat bran-based formulations of *C. membranifaciens* in reduction of blue mold.

Keywords: nutrient sources, *Candida membranifaciens,* formulation, biological control, blue mold.

Introduction

Apple (*Malus domestica* Borkh) is susceptible to various fruit diseases, including gray mold and blue mold. *Botrytis cinera* and *Penicillium expansum* are two critical fungal pathogens causing decay of apples during storage (Mounir *et al*., 2007). Limited control of these diseases is achieved primarily with postharvest synthetic fungicides. However, growing international concern over the excessive use of fungicides and their detrimental effect on the human health and environmental protection have resulted in increasing researches about alternative methods to control post-harvest diseases (Jijakli and Lepoivre, 2004). Treatment of fruit with microbial agents has been demonstrated to be efficient method for control of postharvest decays (Fan and Tian, 2001; Janiesiewics and Roitman, 1998). Some yeasts and bacteria are reported to effectively control various postharvest decays of fruits (Etebarian *et al*., 2005; Gholamnejad *et al*.,

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2009; Piano *et al*., 1997). Several yeasts, such as *Pichia* sp. (Piano *et al*., 1997); *Candida pulcherrima* (Kinay and Yildiz, 2008), *C. sake* CPA-1 (Vinas *et al*., 1998) *Rhodotorula minuta* and *Pichia guilliermondii* (Delen *et al*., 2000) as well as *Pantoea agglomerans*, are reported as effective biocontrol agents against postharvest pathogens (Teixidó *et al*., 2001). Although many researchers have worked on microbial antagonists of postharvest pathogens, little research has been done on the optimization of growth medium for the production of biocontrol agents and results of few researches have been commercialized (Abadias *et al*., 2003). In general, low-cost, high-quality bioproducts are crucial for the future success in alternative control methods of diseases. Mass production of microbial cells and development of a suitable low-cost media are essential steps and key factor in the commercialization of biocontrol agents. A low-cost medium based on cane molasses; a by-product from the sugar industry, could be used at industrial scale and has been developed for *C. sake* (Abadias *et al*., 2000; Abadias *et al*., 2003).

Optimization of growth conditions due to biomass production is very important and takes place under laboratory conditions before up scaling for mass production. Raw materials in the form of food industrial wastes have been channeled towards production of industrial, commercial and pharmaceutical products that include energy, and fine biochemical products (Turker, 2004). The last step to develop a biocontrol product is formulation (Torres *et al*., 2002). The successful delivery of biocontrol agents, shelf life, stability, and effectiveness in commercial conditions depends on the formulation. In the formulation, the carrier is the primary material used to allow a bio-product to be dispersed effectively (Kinay and Yildiz, 2008). The aim of this study is the optimization of biomass production and powder formulation of a promising isolate of *Candida membranifaciens* towards control of blue mold of apple under storage conditions.

Materials and Methods

Micro-organisms

The strains *C. membranifaciens* and *P. expansum* were obtained from Department of plant protection, university of Tehran. The yeast strains were originally isolated from the surface of apple fruits and they could considerably decrease blue mold of apple, caused by *P. expansum* (Gholamnejad *et al*., 2009).

Cultivation experiments

To determine incubation time that supports maximum growth of *C. membranifaciens,* the cultivation experiments were performed using 100-ml volumes of Sabouraud Dextrose Broth (SDB) culture medium in 250 ml flasks. The culture medium was inoculated with 1% (v/v) of inoculum (1×10^9 CFU ml^{-1}), which was prepared from 48 h culture. Flasks were incubated at 30 °C in an orbital shaker at 150 rpm. In this experiment, the growth of *C. membranifaciens* was measured at regular time intervals until 30 h of incubation. The optical density of the samples was measured at 600 nm. Determination of biomass was performed by measuring colony-forming units (Druvefors, 2004).

Determination of carbon sources and nitrogen sources

The effect of carbon and nitrogen sources, as separate and integrated treatments, on production of yeast biomass was studied to determine best conditions for optimal cell production. Thirty four different media were used to determine the best medium for mass cell production of *C. membranifaciens*. Three carbon sources (sugar beet molasses, sugar cane molalsses, and permit powder) and three nitrogen sources (yeast extract, urea and corn steep liquor) were added in the production medium at the final concentration of 4% and 1.2% (w/v), respectively. This experiment was replicated three times in a completely randomized design. The treatments were arranged as described in table 1.

Table 1 Composition of each medium in the present study.

Number	Contents[1]	Number	Contents[1]
1	SBM 4%	18	SBM 2% + PP 2% + CSL 1.2%
2	SCM 4%	19	SCM 2% + PP 2% + CSL 1.2%
3	PP 4%	20	SCM 2% + PP 2% + YE 1.2%
4	CSL 1.2%	21	SCM 2% + PP 2% + urea 1.2%
5	YE 1.2%	22	SBM 2% + PP 2% + CSL 1.2%
6	Urea 1.2%	23	SBM 2% + PP 2% + YE 1.2%
7	SCM 4% + CSL 1.2%	24	SBM 2% + PP 2% + urea 1.2%
8	SCM 4% + YE 1.2%	25	SCM 4% +CSL 0.6% + YE 0.6%
9	SCM 4% + urea 1.2%	26	SCM 4% + CSL 0.6%+urea 0.6%
10	SBM 4% + CSL 1.2%	27	SCM 4% + urea 0.6% + YE 0.6%
11	SBM 4% + YE 1.2%	28	SBM 4% + CSL 0.6% + YE 0.6%
12	SBM 4% + urea 1.2%	29	SBM 4% + CSL 0.6% + urea 0.6%
13	PP 4% + CSL 1.2%	30	SBM 4% + YE 0.6% + urea 0.6%
14	PP 4% + YE 1.2%	31	PP 4% + CSL 0.6% + YE 0.6%
15	PP 4% + Urea 1.2%	32	PP 4% + CSL 0.6% +Urea 0.6%
16	SBM 2% + SCM 2% + CSL 1.2%	33	PP 4% + Urea 0.6% + YE 0.6%
17	SBM 2% + SCM 2% + YE1.2%	34	Sabouraud Dextrose Broth

SBM, sugar beet molasses; PP, permit powder; CSL, corn steep liquor; SCM, sugar cane molasses; YE, yeast extract.

One hundred ml of each medium was dispensed into 250 ml Erlenmeyer flasks. The media were inoculated with cell suspension of 48 h pre-culture of *C. membranifaciens* and incubated at 25 °C for 24 h on rotary shaker at 150 rpm. Un-inoculated media were prepared as control. The yield of yeast in the different test media was recorded after 24 h using serial dilution method. For rapid estimation of biomass concentration, correlation between CFU ml^{-1} with cell turbidity was measured using a spectrophotometric method at OD 600nm.

Biomass production

1ml of the 36-hour pre culture of *C. membranifaciens* was added to the 100 ml of sugar cane molasses-based medium in 250 ml flask and then was shaken for 24 hours. The biomass was harvested by centrifugation at 3000 rpm for 10 min. Harvested cells resuspended in distilled water. Cell concentrations were determined with a hemocytometer. The centrifuged cells were used in the formulation.

Preparation of formulation

Talc, kaolin, rice bran and wheat bran were used as carriers for powder formulation of yeast-cell suspension (1×10^9 CFU ml^{-1}). Biomass of yeast strains were incorporated into glycerol (10% v/v) and sodium alginate (1.5% w/v). This suspension was added to carriers. The resulting mixture was spread on a sterile bench and air-dried at laboratory conditions for 1 day. After drying, the formulations were powdered in a Warring blender and stored in sterile tubes at 24 °C and 4 °C for 180 days.

Viability tests

Viability of the yeast cells in powder formulations stored at 4 °C and 24 °C was determined every month during six month

storage. Colony forming units (CFU) were counted after 2 days of incubation at 24 °C and calculated as CFU g^{-1} of each formulation.

Efficacy of formulations against *P. expansum* on apple fruit

The various formulations were assessed for their efficacy in controlling *P. expansum* in storage conditions. Apples cultivar Golden Delicious that had been harvested at commercial maturity was used for inoculation tests. The apples were surface sterilized. Dried fruits were wounded by removing plugs of (3 × 5 mm) from the surface. Each apple was wounded three times halfway between the calyx and the stem end. All wounds were treated with 40 µl of each formulations suspension. After 24 hours wounds were inoculated with 20 µl of *P. expansum* (10^5 spore ml^{-1}). To evaluate the effect of treatments on development of decay, fruits were treated with pathogen plus carriers, carriers without pathogen, and yeast antagonist as stand-alone treatments or in combination as described previously. The apples were placed in sterile boxes and stored at 4 °C. Lesion diameter was determined after 6 weeks. For each apple, the reduction in lesion diameter was calculated.

Results

Kinetic of biomass production of *C. membranifaciens*

The kinetics of the yeast growth is shown in figure 1. Our results indicated that after 24 h incubation at 30 ºC, maximum rate of yeast growth appeared.

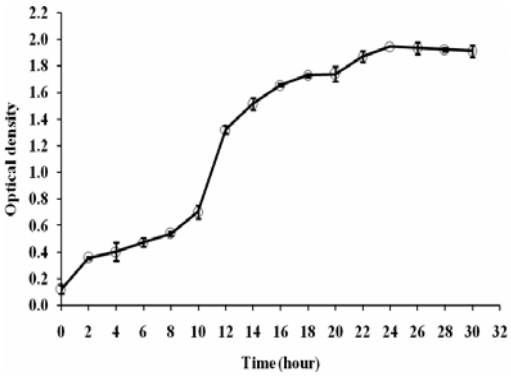

Figure 1 Kinetic of biomass production of *Candida membranifaciens* in SDB medium after 30 h of incubation at 30 ºC.

Determination of carbon sources and nitrogen sources

The growths of *C. membranifaciens* in different media are presented in figure 2.

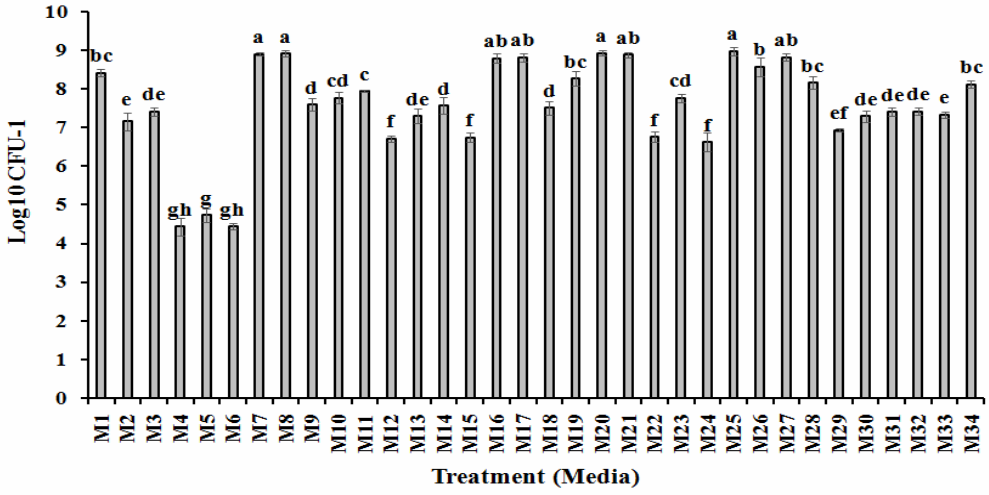

Figure 2 Growth of *Candida membranifaciens* in different media with various carbon and nitrogen sources. Means followed by the same letter are not significantly different (Duncan's multiple range test, $P < 0.01$).

Yeast growth in media containing sugar cane molasses was significantly higher than those grown in media without sugar cane molasses. Low levels of yeast growth were recorded in media that were supplemented with permeate powder as carbon sources. Growth in permeate powder, sugar beet molasses and sugar cane molasses yielded biomass levels 9×10^5 CFU ml^{-1}, 1×10^6 CFU ml^{-1} and 5×10^7 CFU ml^{-1} respectively. Biomass production was increased by adding nitrogen sources to media. Yeast extract as nitrogen source produced more biomass compared to Urea and CSL 4%. The best growth was obtained in sugar cane medium plus yeast extract and CSL. The final growth of *C. membranifaciens* in sugar cane molasses based media was similar to SDB.

The shelf life of yeast formulations

Final viable cells per mg of formulation are a critical factor in formulating yeast cells. A strong effect of carriers on the survival of yeast isolate was observed in powder formulation. When wheat bran was used as carrier, yeast cells demonstrated greatest viability during the experimental period over six month 10^9 CFU ml^{-1}. Results of storage at 4 °C showed that viable counts of yeast cells in talc and kaolin based formulation decreased up to 2 orders of magnitude after six months. After six months of storage at 4 °C, a final viable count of *C. membranifaciens* in Talc and Kaolin and wheat bran-based formulations was 8.7×10^8, 2.6×10^7 and 2.1×10^{10} CFU g^{-1} respectively. Viability of formulated yeast in different carriers differed significantly ($p < 0.01$). Kaolin and Talc as carriers could gradually reduce the viability of *C. membranifaciens* in formulations while, wheat bran, increased the number of viable cells in formulations up to 4 months and then viable cells were gradually reduced. In spite of that, after six months of storage the number of viable cells in formulations that wheat bran was used as carrier was more than the initial cells number used in the formulation (Fig. 3).

In general, storage at 4 °C gave the highest number of viable cells for all formulations examined, while storage at 24 °C reduced the survival for all formulations.

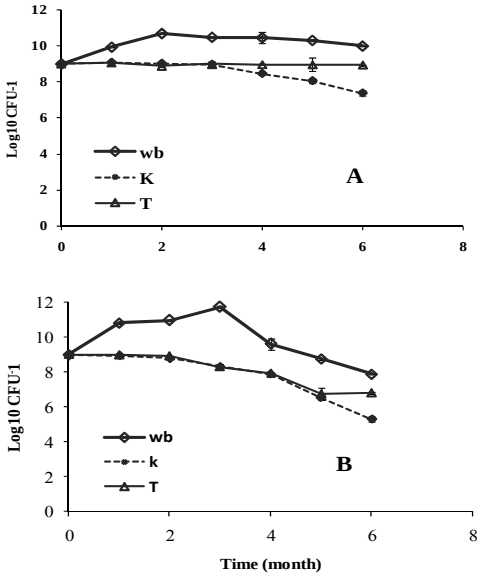

Figure 3 Viability (CFU g^{-1}) of yeast isolate within various formulations (Wb; Wheat bran based formulation, K; Kaolin based formulation and, T; Talc based formulation) during shelf life period over 6 months. They were stored in the dark at 24 ºC (B) and 4 ºC (A). Error bars indicate standard deviations (SD) of three replications.

Effectiveness of yeast formulations for the control of blue mold on apple

Efficacy of the various formulations of *C. membranifaciens* was evaluated for controlling blue mold of apple *P. expansum* at storage conditions. Statistically, the lesion diameters caused by *P. expansum* were significantly reduced by all formulations of *C. membranifaciens*. There was no significant difference among Talc-based, Kaolin-based and Wheat bran-based formulations of *C. membranifaciens* in reduction of blue mold. In addition, carriers used in formulation without yeast cells did not reduce lesion diameter and were similar to untreated control (Table 2).

Table 2 Decay area on Golden delicious apples inoculated with *Penicillium expansum* challenged with formulations of yeast and incubated for 45 days at 4 ºC.

Treatment[1]	Decay (mm^2)[2]	Treatment	Decay (mm^2) [2]
T + pathogen	485.80 b	Fresh cell of yeast + pathogen	321.65c
K + pathogen	523.73 b	T	0.00 d
WB + Pathogen	477.03b	K	0.00 d
T + Pathogen	3185.87 a	WB	0.00 d
K + Pathogen	3156.25 a	Fresh cells of yeast	0.00 d
WB + Pathogen	3263.00 a	Control (Without pathogen)	0.00 d
Control (Pathogen)	3255.77 a		

[1] T: Talc based formulation; K: Kaolin based formulation; WB: wheat bran based formulation.
[2] Means followed by different letter in column are significantly different (Duncan's multiple range test, $P < 0.01$).

Discussion

Our strategy for optimizing nutritional conditions for the mass production of biocontrol yeast isolate in liquid culture is based on developing a medium which supports high cell density production as well as low cost. Media optimization is not only a means of improving mass cell production, but also is a very important cost factor (Rivas *et al*., 2004).

Inexpensive sources can be found in form of industrial waste-or by-products. Examples are Molasses, Soybean, hydrolyzed wheat flour; cheese whey and corn steep liquor (Oh *et al*., 2004, Wee *et al*., 2006).

Molasses is a by-product of sugar production and CSL contains vitamins and minerals. They have been used successfully for fermentations. Moreover, many papers have reported that molasses and CSL are economical sources for fermentation process (Kim *et al*., 2007).

Our results indicated that the efficacy of sugar cane molasses as a carbon source was better than sugar beet molasses and Permeate powder. It seems that these differences reflect their components. It is well-known that lactose is main sugar of cheese whey while sugar cane molasses contain 44% sucrose, 13% fructose, 10% glucose and sugar beet molasses contain 66%sucrose, 1% fructose and 1% glucose (Steg and Van der Meer, 1985). In the consumption studies, it could be seen that *Candida* cells prefer the use of glucose and fructose more than sucrose. Glucose is considered the main carbon source by all microorganisms because of its size, rapid uptake, utilization and cellular energy conversion. Once glucose and fructose were consumed, *Candida* cells started to assimilate sucrose (Abadias *et al.,* 2003). It can be validation of our result about superiority of sugar cane molasses in comparison with other Carbone sources that were used in this study. Low levels of *C. membranifaciens* in permeate powder based media suggests that the strain is not adapted to ferment lactose.

Formulation is an important step in developing a biocontrol product. The successful delivery of biocontrol agents, shelf life or stability, and effectiveness in commercial conditions depend on the formulation. In the formulations the carriers are the primary material means that allow a bioproduct to be dispersed effectively (Kinay and Yildiz, 2008).

In this study, an inexpensive, convenient and effective method to develop stable formulation of *C. membranifaciens* was investigated by using organic and inorganic materials as a carrier. Our results showed that yeast cells could survive on organic and inorganic carriers for more than six months. The ability of various carriers to keep the yeast cells viability for six months at 4 °C and 24 °C was significantly different. These results indicated that yeast cells can remain dormant and survive in the formulation and inorganic carriers. Using inorganic carriers (Talc, Kaolin) indicated that viable yeast cells decrease during storage; nonetheless were relatively stable in viable cell counts throughout the investigated period. The maximum yeast cell

life was observed in talc. Other researchers pointed out that talc is the best carrier in the experimental formulations in terms of maximum shelf life and survival of antagonist's cells (Kinay and Yildiz, 2008; Kloepper and Schroth, 1981).In our study, wheat bran caused an increase in cell counts (10-fold) at the end of the 6-month period. Propagule density increased more than 100-fold within 60 days and remained high even after six months. Rice bran and wheat bran are acknowledged rich resources of foodstuffs. Contact of cells of yeast isolates with a food base enables their germination in different substrates. This is in accordance with the findings of Cozzi and Gasoni (1997) who indicated that isolate TH-1 of *Trichoderma harizanum* proliferated in peat and vermiculite based formulations. They suggested that probably increasing more than 100-fold in propagule density of *T. harizanum* formulations was related to substrates food content.

However, an ideal situation for any microbial agent is that the formulated strain stays dormant to provide the strain stability in the final product throughout the circulation period (Chung *et al.*, 2007).

Also, regardless of our formulations, we considered that survival and germination of yeast isolate in formulations was a function of yeast species and of carriers and additives. These results suggested that talc and kaolin carriers were very effective in maintaining the dormancy of the yeast cells for at least 6 months, while wheat bran stimulated the germination and hence the proliferation of the vegetative cells.

Since low temperatures are imperative for prolonged storage, it is important that the effectiveness of antagonists be tested under prevalent storage conditions rather than at ambient temperature. (Spotts and Sanderson, 1994).

The best control of blue mold caused by *P. expansum* inoculated at a concentration of 1×10^5 CFU ml^{-1} was achieved by application of *Aureobasidium pullulans* at a concentration of 1×10^8 CFU ml^{-1} (Arul, 1994; Mounir *et al.*, 2007). Our results demonstrated that use of yeast formulations with different carriers could effectively reduce blue mold of apple.

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تولید بیوماس و فرمولاسیون مخمر بیوکنترلی *Candida membranifaciens*

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چکیده: در این مطالعه تأثیر منابع کربن و نیتروژن مختلف روی تولید بیوماس مخمر و هم‌چنین تأثیر فرمولاسیون‌های مختلف مخمر بیوکنترلی *Candida membranifaciens* در کاهش بیماری کپک آبی سیب مورد بررسی قرار گرفت. محیط کشت حاوی ملاس نیشکر به‌عنوان منبع کربن در مقایسه با محیط کشت حاوی آب پنیر کارایی بالاتری در تولید بیوماس داشت. عصاره مخمر به‌عنوان منبع نیتروژن نسبت به اوره و عصاره ذرت خیسانده شده (CSL) در تولید بیوماس سلولی تأثیر بیش‌تری داشت. بهترین رشد زمانی حاصل شد که از ملاس نیشکر و عصاره مخمر و CSL مورد استفاده قرار گرفت. تعداد سلول‌های زنده در فرمولاسیون‌های مبتنی بر پودر تالک و کائولین به‌تدریج کاهش یافت. درحالی‌که در فرمولاسیون مبتنی بر سبوس گندم تا چهار ماه تعداد سلول‌های زنده افزایش پیدا کرد و سپس شروع به کاهش نمود. در حالت کلی فرمولاسیون‌های نگهداری شده در دمای ۴ درجه سلسیوس طول عمر بیش‌تری نسبت به نمونه‌های نگهداری شده در دمای ۲۴ درجه سلسیوس داشتند. فرمولاسیون‌های تهیه شده از *C. membranifaciens*، برای کنترل *Penicillium expansum* در شرایط انبار مورد ارزیابی قرار گرفتند. فرمولاسیون‌های مختلف *C. membranifaciens* از جمله سه نوع فرمولاسیون مبتنی بر تالک، کائولین و سبوس گندم اختلاف معنی‌داری در کاهش بیماری کپک آبی نشان ندادند.

واژگان کلیدی: منابع غذایی، فرمولاسیون، کنترل بیولوژک، کپک آبی، *Candida membranifaciens*