ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Production and Optimization of *Rhodotorula glutinis* for biocontrolling of Strawberry Blight.

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ABSTRACT

Biocontrol agents represent an alternative or supplement to chemicals for disease control. *Rhodotorula glutinis* was evaluated for its activity in reducing blight and gray mould diseases of strawberry caused by *Botrytis cinerea* and *Phomopsis obscurans in vitro* and *in vivo*. Optimization of biomass and metabolite production were determined using some cultural conditions like different incubation time in hours, pH, carbon sources , concentrations and nitrogen source. Yeast was produced hydrolysis enzymes and siderophores (Rhodotorulic acid) when grown in a medium with excess fructose as carbon source and soybean as nitrogen source . The stabilization technique was have evaluated using liquid formulations . Yeast showed high preserve viability of when stored at 4°C for six months. Preharvest application of liquid formulation of certain microbial isolate provided an effective control of leaves blight as well as fruit gray mould disease pre and protect fruit during storage than the tested fungicide at the recommended levels.

Keywords: Antifungal , *Botrytis cinerea* , *Phomopsis obscurans* , Optimization , *Rhodotorula glutinis* , strawberry blight and yeast

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ISSN: 0975-8585

INTRODUCTION

Strawberry (*Fragaria ananassa* L.) production in Egypt is very minor compared to the principal fruits. Leaf blight, caused by the fungus *Phomopsis obscurans*, is an important summertime disease because so few varieties have adequate resistance to it. (Haggag, Wafaa, 2009). Grey mould diseases caused by *Botrytis cinerea* are major flower, fruit, and foliar in Egypt (Haggag, Wafaa, 2008) pathogens of field-grown strawberries and can affect almost every variety in Egypt. Postharvest losses of fruit and vegetables can reach very high values, representing more than 25% of the total production in industrialized countries and more than 50% in developing countries . Exploring biological fungicides from microbial metabolites is an important research direction of fungicides and has been received more attention (Haggag, Wafaa, 2013). Yeasts of the genus Rhodotorula (particularly *Rhodotorula glutinis*) have been reported as good biocontrol agent against *Penicillium expansum,* in apples and pears (and against *Botrytis cinerea* in strawberry (Helbig 2001) when preharvest and/or postharvest applications are made. The production of hydrolysis enzymes, rhodotorulic acid, a siderophore, by the yeast *Rhodotorula glutinis* contributes greatly to biocontrol of blue mold on apples and the addition of some nutrients, e.g. proline, and manipulating pH stimulated production of the siderophore (Calvente *et al*., 2001) and carotenoids (Saenge *et al.,* 2011). The important characteristics of yeast cell wall are referred to as mannans, mannan conjugates, high water solubility; broadspectrum of biological activity, low toxicity, stability and anti-mutagenic effects via different modes of action (Dae *et al*., 2001 and Haggag, Wafaa, 2013). Beta-carotene is an excellent antioxidant (Bhosale, and Gadre, 2001). Enhancing biocontrol using these manipulations may be possible if they do not benefit the growth of the pathogen and outweigh the benefits of higher siderophore production by the antagonist. In order to obtain semi-commercial amounts of the microbial agent, it is necessary to scale-up the production process, at least to the level of pilot plant, using a low cost culture medium. To our knowledge, there are no papers that have addressed the scale-up aspects of any *Rhodotorula* fermentation process. The levels of fermentation are related to fermentation time, ventilation, temperature, initial pH etc. Biocontrol strain fermentation is greatly influenced by the combination of media components and culture conditions in laboratory or industrial fermentation. Fermentation time is a very important factor, which affect the yield and quality of metabolites. The microbial agent should be formulated as a product having long storage stability, of at least 6 months (Janisiewicz and Korsten 2002). In this study, we tried to optimize fermentation condition of yeast *Rhodotorula glutinis* and determine the optimal combination of the medium composition , culture conditions optimization and fermentation , formulation and shelf life to improve the antifungal productivity, enhance the control effect of blight diseases caused by *Botrytis cinerea* and *Phomopsis obscuran*s under field and storage conditions in two harvest seasons.

MATERIALS AND METHODS

Culture Conditions and Medium

Yeast *Rhodotorula glutinis* was isolated from Strawberry plants and stored in the laboratory (Haggag Wafaa *et al.* 2005) . Isolate was grown on YPD medium containing 2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract. *B. cinerea* and *P. obscurans* were isolated from diseased Strawberry grown in Boheria governorate. *Botrytis cinerea and Phomopsis* on PDA. They were stored at 4°C until required. These isolates were maintained at –20°C as spore suspensions in 20% glycerol and were activated in potato dextrose agar for 7 days.

In vitro antagonist assays

Petri plates (90 mm diameter), preinoculated 48 h before – to compensate the velocity growth differences – with 1 cm2 plug of a fresh culture of *B. cinerea* and *P. obscurans* (grown on PDA), were inoculated with a yeast suspension containing $1 \cdot 10^8$ CFU ml)1. After the yeast was inoculated, the plates were incubated for 48–72 h at 29 ± 1_C. In vitro antagonistic assays were conducted in two manners: (a) The fungal pathogen and the antagonistic yeast were inoculated at the opposite extremes of the 90-mm diameter Petri plate with PDA medium. (b) The yeast was plated in order to get a circumference growth and the fungus was inoculated in the centre of the plate. The control plate was only inoculated with the fungal pathogen either in the centre or at the extreme of the Petri plate. After incubation for 24–72 h, plates were evaluated.

Antifungal Activity Assay

Antifungal activity was measured by assaying the mycelium growth inhibition rate of *B. cinerea* and *P. obscuran*s. Briefly, 1mL sterile fermentation filtrate mixed with 9 mL PDA was replaced in a dish or without sterile fermentation filtrate as control. After the medium solidifying we inoculated *B. cinerea* and *P. obscuran*s mycelium discs which as incubated for 3d on the dish. The dishes were incubated in the dark at 28°C in incubator for 72 h . The percentage of inhibition was calculated from the following equation:

Inhibition (%) = [(growth diameter in the control sample – growth diameter in the sample with treated yeast) × 100]/growth diameter in the control sample.

The experiment was repeated twice in triplicates.

Measurements of *R. glutinis* cell growth protein and biopolymers production

Cell growth, protein and biopolymers produce by *R. glutinis* were measured in culture filtrates after four and seven days of incubation. Cells growth were monitored by measuring the difference in protein contents between culture broth and culture supernatant. Cells growth were determined by measuring the optical density at 610 nm. Protein content in the supernatant was determined at 595 nm by the method of Bradford (1976) using bovine serum albumin as a standard.

Enzymes activities

Activities of hydrolysis enzymes were assessed in culture filtrates of the tested yeast isolates over 7 days using a Labsystems Uniskan II microtiter plate spectrophotometer. Exo-glucanase activity was detonated using glucose oxidase-O-dianisidine reaction (Sigma Chemicals, glucose determination kit 510-A) which specifically measures glucose produced from laminarin hydrolysis. ß-1,3-Glucanase activity was assayed by incubating 0.2 ml of culture filtrate in 50 mM potassium acetate buffer (pH 5.5) with 50 µl of enzyme solution appropriately diluted in the same buffer. Reaction mixtures were incubated at 37°C for 30 min and were stopped by boiling for 5 min. One unit of ß-1,3-glucanase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar equivalents (expressed as glucose) per min under the standard assay conditions. The activity of exo-chitinase, was measured as the release of N-acetylglucoseamine from chitin and one unit (U) of enzyme activity was defined as the amount of enzyme that release 1 µmol of reducing groups/ min/ ml of the filtrate (Bradford, 1976).

Siderophores assay

The amount of siderophores (Rhodotorulic acid) excreted into the culture medium was determined by spectrophotometer. Concentration was calculated using absorption maximum and the molar absorption coefficient (max; 400 nm) according to the method of (Meyer and Abdallah, 1978).

Batch Fermentation

Incubation period

The optimization of composition of incubation period, and cultural conditions was carried out based on stepwise modification of the governing parameters for antifungal production. The cultures were transferred to seed broth (200 mL of Medium) contained in a 500 mL Erlenmeyer flask and incubated at 30°C on a rotary shaker (175 rpm) for 6-8 hours. A 500 mL Erlenmeyer flask containing 200 mL of the same seed medium was incubated as specified above. The seed culture was transferred to a 5 liter fermenter containing each one 3.5 liter of the three liquid media

Determination of biomass by cell dry weight

Bioagent growth was estimated directly by spectrophotometric measurement of the OD600 (Amax) using a PM 2A spectrophotometer and dry biomass concentration (bmax). Changing the pH 3 to 10 in the production medium the effect of pH was observed. The effect of cultivation temperature on the antifungal

production was examined at different temperatures starting from 25 to 60^{0}C with 5^{0}C intervals. The supernatants were discarded and the centrifugation tubes were dried in an oven at 100°C till constant weights were obtained. The weight of the dried cells was measured by calculation of the difference between the weight of centrifugation tubes before and after dryness.

Effect of different carbon sources and concentrations

The tested carbon source was added at a concentration equivalent in carbon content to that of glucose (10 g/L). The tested carbon sources were (D-glucose, D-fructose, sucrose, galactose, Starch, Cellulose, treated molasses and none treated). Erlenmeyer flasks (250 ml) containing 50 ml of the medium devoid of its own carbon source and containing the equivalent amount of other carbon sources were prepared, inoculated and incubated as described in described above. At the end of the incubation period, samples were removed for determination of biomass (optical density and dry weight), residual substrate and inhibition zone.

Flasks (250 ml) with 50 ml aliquots of the medium containing different concentrations of the carbon source that showed promising results were prepared. These carbon source was surose (5-30 g/L). The flasks were terminally sterilized, inoculated and incubated as described above. At the end of the incubation period, samples were removed for determination of biomass (optical density and dry weight), residual substrate and inhibition zone.

Effect of different nitrogen sources

This was studied by replacing sodium nitrate of medium with other tested nitrogen sources. The tested nitrogen source was added at a concentration equivalent in nitrogen content to that of sodium nitrate (2.5 g/L). The nitrogen sours were Peptone + yeast extract**;** Pepton + trypton**;** Peptone+ soyabean; Yeast extract+ trypton**;** Yeast extract + soyabean; Trypton+ soyabean**;** Soyabean + trypton; Pepton**;** Yeast extract; Trypton; Soyabean. In case of urea, a stock solution was prepared; filter sterilized (0.22 µm) and then an aliquot containing the required amount of urea was incorporated aseptically. At the end of the incubation period, samples were removed for determination of biomass (optical density and dry weight), residual substrate and inhibition zone.

Studying the time course of enzymes production by isolate in medium under shaking condition.

Erlenmeyer flasks containing the previously mentioned medium were prepared, inoculated and incubated as described above. Over an incubation period of 7 days, one flask was removed every 8h., determination of biomass (optical density and dry weight), residual substrate , inhibition zone and metabolites products .

Formulation

The formulations of *R. glutinis* were prepared using 0.01% as glycerol oil , soybean oil, polysaccharide as methylcellulose (CMC), D-glucose at 0. 1% concentration. The liquid formulations were evaluated initially during storage periods at 4ºC temperature. The percent of survival bacteria were measured during three months.

R. glutinis suspension was centrifuged for 20 min at 6.000 rpm. *R. glutinis* pellet was resuspended in 0.1M $MgSO_4$ in a 1:1 (w/v) ratio and then combined with either 15% (v/v) glycerol which was included to stabilize the bacterial cells. Thereafter, the suspension was mixed with an equal volume of autoclaved 1.5% Na-Alginate . The *R. glutinis* Na Alginate mixture was combined with sterilized starch at the ratio 1:4 (v/v) (11). A wetting agent (Ca-lignosulphate) was added (7%, w/w) to the mixture. The resulting mixture was spread thinly over a glass sheet and air dried in a laminar air-flow cabinet at 24ºC for 1 hour to form a slightly moistened powder (15% moisture content). After drying, the *R. glutinis* formulation was powdered in a Waring Blendor and stored in glass bottles with lids as small volumes . The Starch -based formulations of strain, were stored at 4ºC for 90 days. Survival of *R. glutinis* in the formulations was assessed at 30 day intervals by a dilution plate method . There were three replications for each analysis.

Field application

Two successive experiment were conducted at El-Kanater El-Khaireia, Qalubeia governorate, Egypt to evaluate the effect liquid formulation of biological control agents on controlling of strawberry blight diseases cv. Selva, highly susceptible to diseases. Transplanting were planted in rows spaced 50 cm between rows and 25 cm between plants in mid-October. After planting, water and fertilizer were provided twice weekly thoroughly drip irrigation. The liquid suspension of *R. glutinis* was collected and adjusted at the concentration of 5 X 10^5 colony forming unit. The formulation was prepared using 0.01% glycerol oil and D-glucose at 0. 1% concentration. Treatments were arranged in completely randomized block, design with 10 replicates per treatment and 1000 plants per replicate. The results were expressed as attack level/attack degree (AL) on fruits and yield and compared to standard (Redomil–plus at 2g/l) and untreated check. All treatments were applied as foliar application on strawberry plants after 50 and 70 days of transplanting meanwhile, fungicide every 15 days up to 120 days of planting .

Pre-harvest evaluations for Botrytis Fruit Rot

All fruit were harvested twice a week, but only graded once a week. The percentage of infection and severity were measured continuously during growth period.

Postharvest evaluations for *Botrytis* Fruit Rot

Three days after second application with *R. glutinis*, strawberry fruits were harvest and divided into two groups , first stored under natural infection and second stored under artificial infection to study their efficacy against gray mould disease of strawberry fruits.

Effect of *R. glutinis* on gray mould diseases of strawberry fruits under natural infection

Strawberry fruits were stored under natural infection (18± 1˚C) for 7 days (Shelf life). Treated fruits were stored inside carton trays (40x25x10cm). All trays were stored at 18± 1˚C for 7 days**.** Gray mould disease was recorded after 7 days of storage

Effect of *R. glutinis* on gray mould diseases of strawberry fruits under artificial infection

Plates were incubated at 18± 1˚C and spores suspension (10^6 spores / ml) of *B. cinerea* were prepared. Fruit inoculation was carried out by spraying fruits with spore suspension (10^6 spores/ml) of *B. cinerea* then air dried at room temperature 23-25C°. Treated – inoculated fruits were stored inside carton trays (40x25x10cm). All trays were stored at 18± 1˚C for 7 days. The percentage of disease incidence and rotted tissue part was recorded after 7 days of storage .

Phomopsis Blight Disease

Leaf blight scale from 1.0 to 6.0 according to Louws, (2007) was modified and used .Disease was recorded up to 100 days of planting.

Effect of *R. glutinis* on some vegetative characters and yield of strawberry plants under field conditions

Number of leaves, weight / plant were determined after 90 days of planting. Accumulated strawberry yield (Ton / feddan) was determined.

Statistical analyses

The effects of the treatments on disease severity were analyzed by using Duncan Multiple Range Test (SPSS software 16).

ISSN: 0975-8585

RESULTS

In vitro bioassays

On PDA medium, large inhibition or clearance zones were observed in the assays conducted of *R. glutinis* against *Botrytis* and *Phomopsis* (Fig. 1). Results in Fig**. (1**) obtained that filtrates of *R. glutinis* give significant effect on reducing the mycelial growth of *Botrytis* and *Phomopsis,* Respectively. Cell growth, and protein concentrations of *R. glutinis* production in liquid medium, are recorded after four days . *R. glutinis* tend to produce high levels of protein (Fig.2).

Enzymes assays

The general ability of tested *R. glutinis* to produce secondary metabolites include hydrolysis enzymes were determined (Fig. 3). Exochitinase and β-1,3- glucanase, appeared to be conman metabolites. *R. glutinis* produced the highest amounts of all three hydrolysis enzymes.

Siderophores assay

The amount of siderophores excreted into the culture medium was determined by spectrophotometer. Final siderophore (Rhodotorulic acid) concentration achieved was almost 0.45 mM/L(Fig. 3).

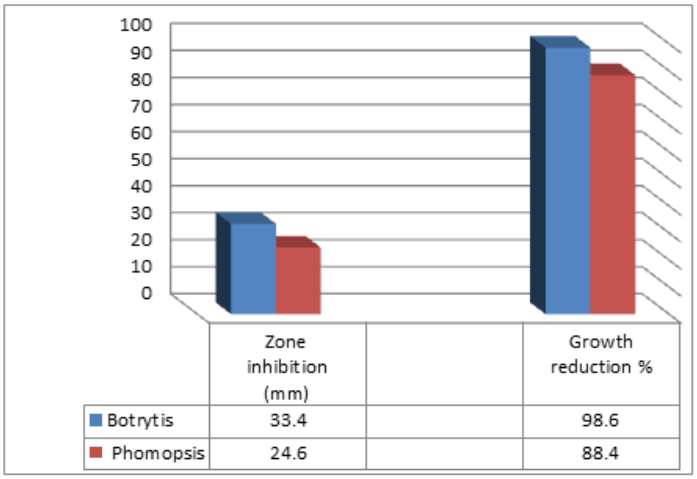

Figure 1: *In vitro* antagonist study of *R . glutinis* against *Botrytis* and *Phomopsis*

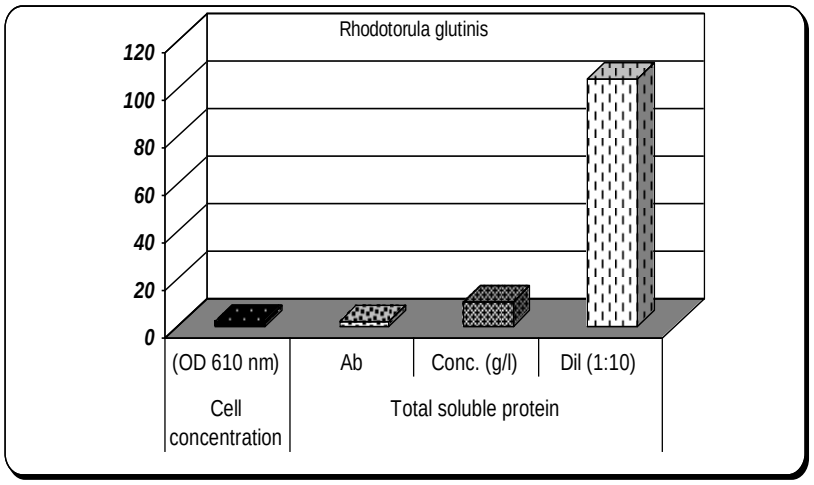

Figure 2: Growth and total soluble protein produce by *R . glutinis*

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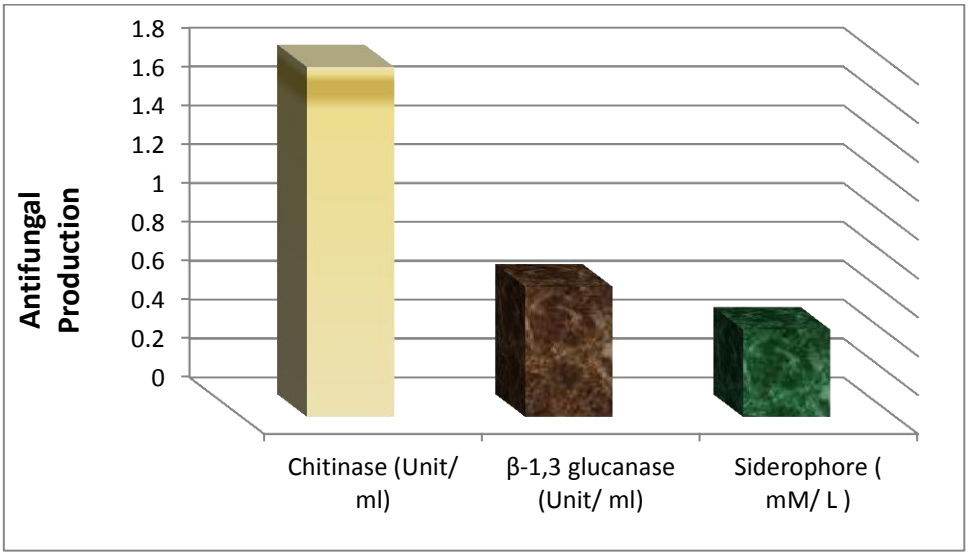

Figure 3: Hydrolysis Enzymes and siderophore produce by *R. glutinis*

Production and optimization

The time course for optical density shows that the maximum value is obtained at 40 hrs incubation time, and then we reach a plateau. This is explained in the residual substrate curve as we notice that a gradual consumption of glucose occurs till 40 hrs then the glucose concentration remains constant for the rest of incubation time. Dry weight increases with time until it reaches its maximum at 36 hrs then decreases slightly with time. In the inhibition zone curve, *Botrytis* follow the same pattern whereas the inhibition zone increases with incubation period till it reaches its maximum at 48 hrs**.** Siderophore production increased radically from the beginning of incubation time till 48 hrs then decreased. β,1-3 glucanase activity gradually increased for up to 48 h of fermentation in both strains and decreased thereafter. From all these, we deduce that ,the maximum incubation period for the growth of *Rhodotorula* is 40 hrs (Fig.4).

From the experimental results it was noticed that by using different types of carbon source, the production of Chitinase and B,1-3 glucanase (unit/ ml) were maximum with glucose and fractose as carbon sources. Also, the zone inhibition of *Botrytis cinerea* was maximum after 48 h incubation with glucose and fructose as carbon sources . So, the fructose and glucose were chosen as an optimum carbon sources for *R. glutinis* (Fig.5). By using different concentration of glucose and fructose for the growth of *Rodotorula*, the dry weight concentration increased after 24 h with increasing the glucose and fructose concentrations till 20 g/l then decreased. Also, the production of B,1-3 glucanase (unit/ ml) and Chitinase increased after 48 h by increasing the glucose concentration till 20 g/l then decreased again. The zone inhibition of Botrytis cinerea increased gradually by increasing the glucose concentration till 20 g/l then remained almost constant. By comparing the results at 20 g/l concentration for glucose and fructose after 48 h it was found that the higher results were obtained using 20 g/l glucose concentration . From the previous observations, 20 g/l was chosen as the optimum glucose concentration(Fig.6).

By using different types of nitrogen source for the growth of *Rodotorula* it was noticed that the maximum biomass growth was obtained using soyabean as nitrogen source while the maximum zone inhibition for *Botrytis cinerea* and maximum production of Chitinase were obtained with peptone and soyabean in equal ratio in the media with a reasonably high dry weight. So, the optimum nitrogen source chosen was an equal ratio of peptone and soyabean (Fig.7).

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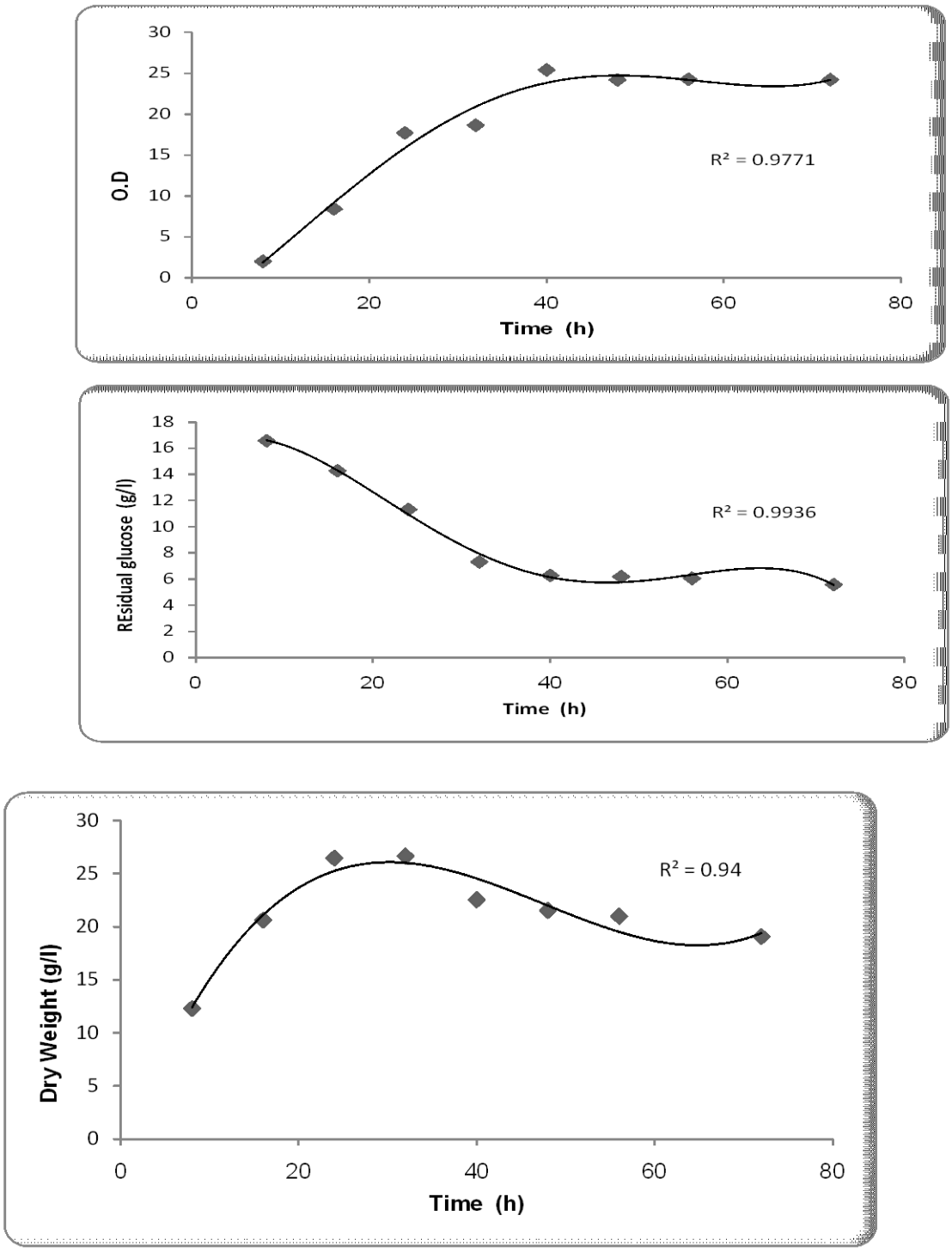

Figure 4: Time course of growth and antifungal metabolite production by *R. glutinis*

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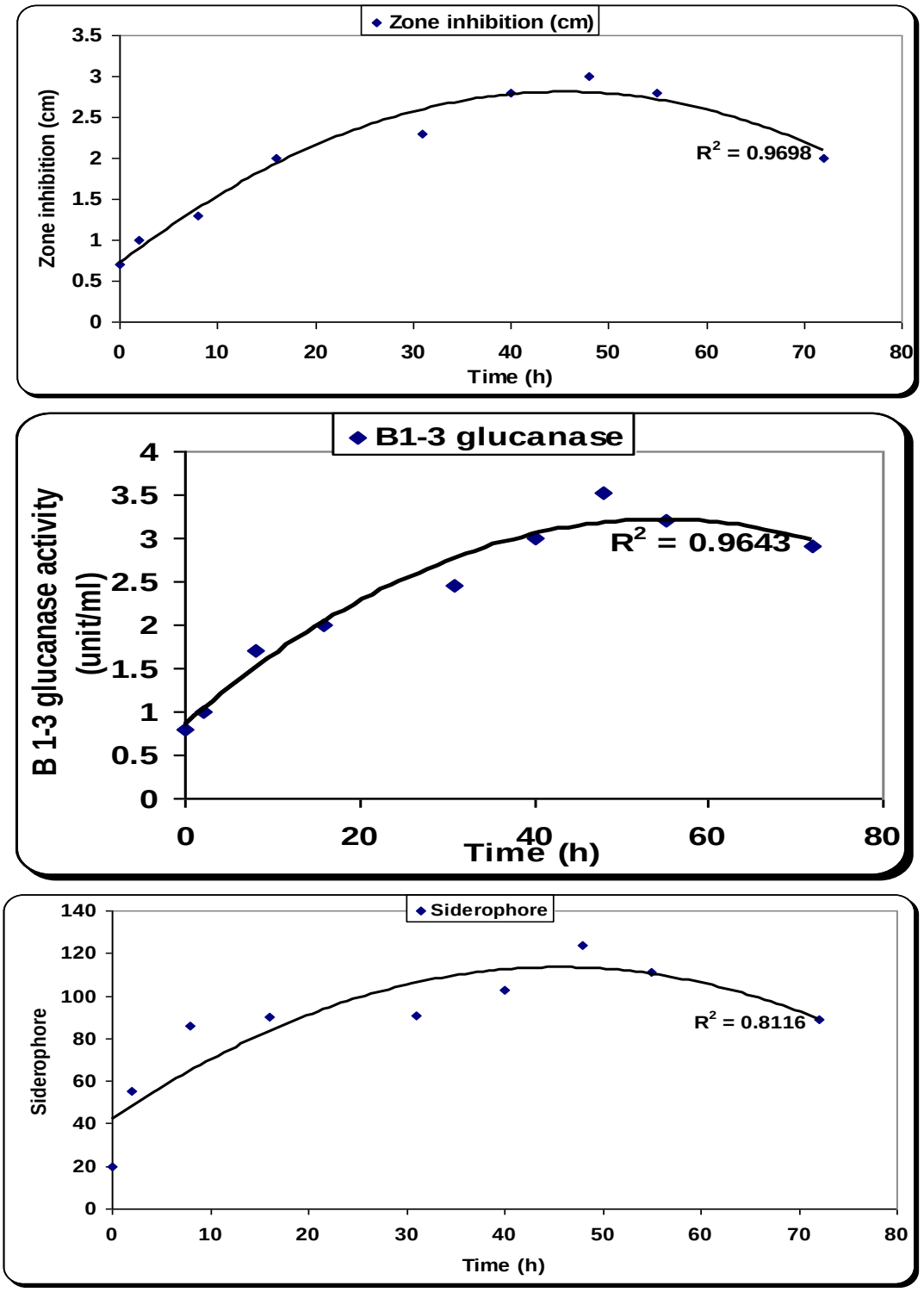

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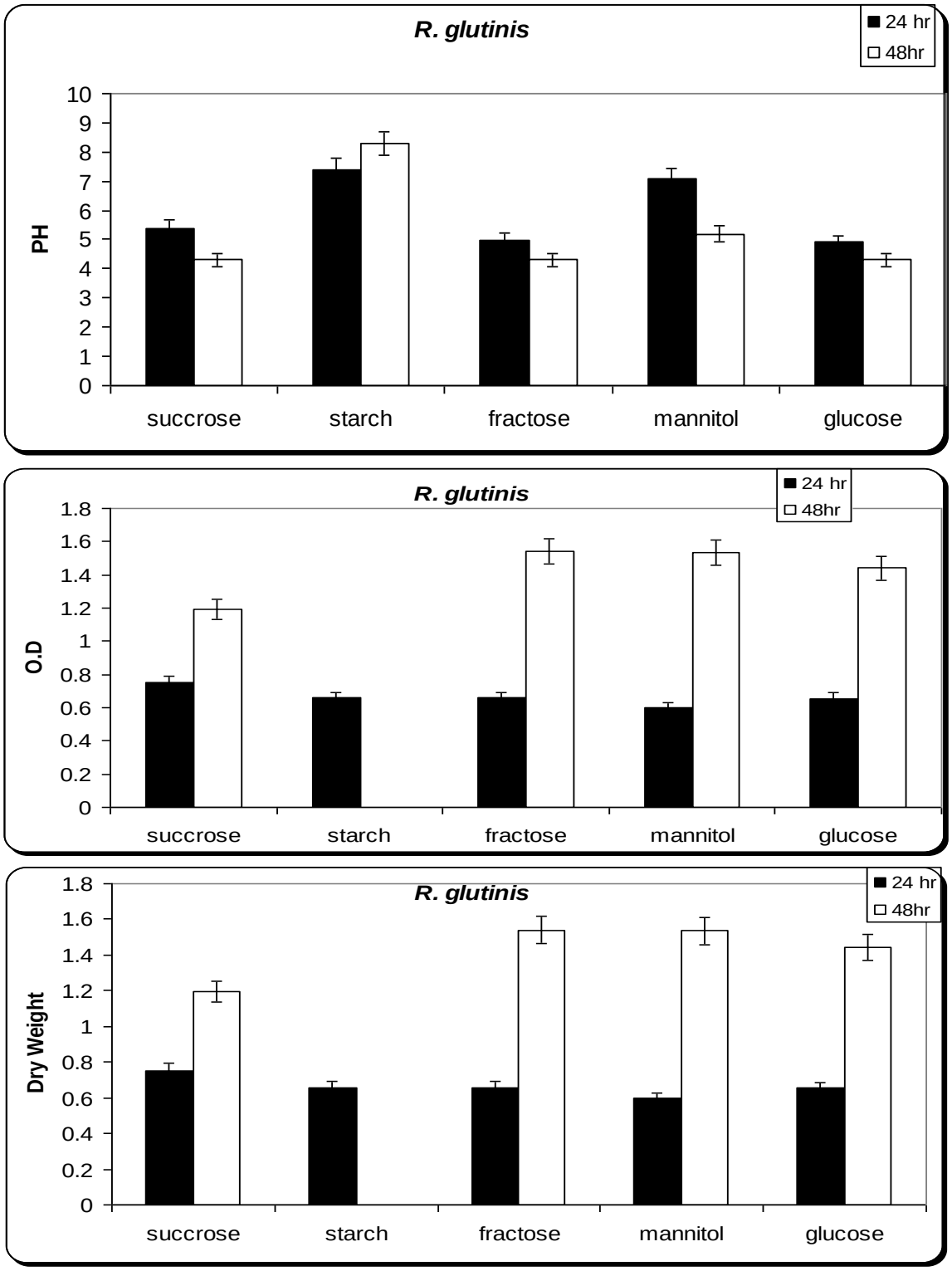

Figure 5: Effect of different carbon sources on the growth, inhibition activity and production of antifungal of *R. glutinis*

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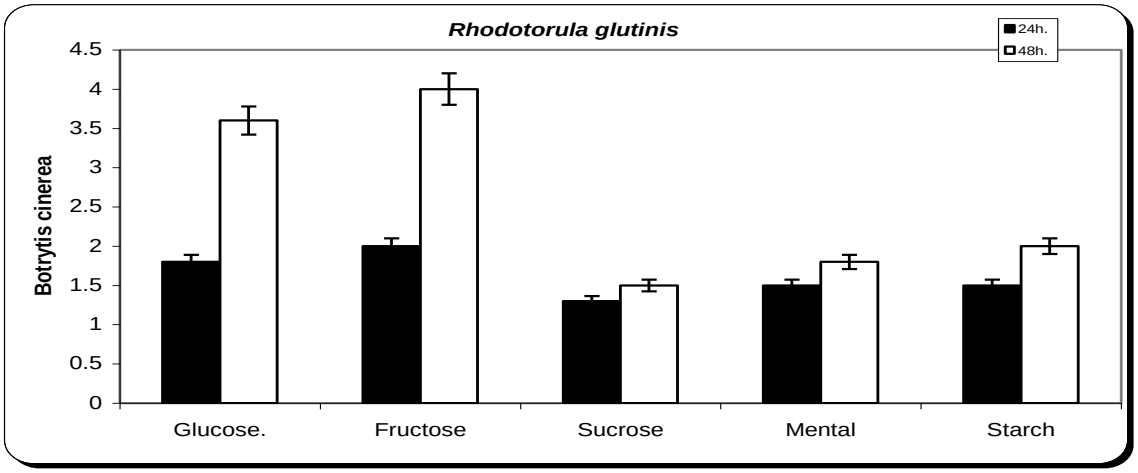

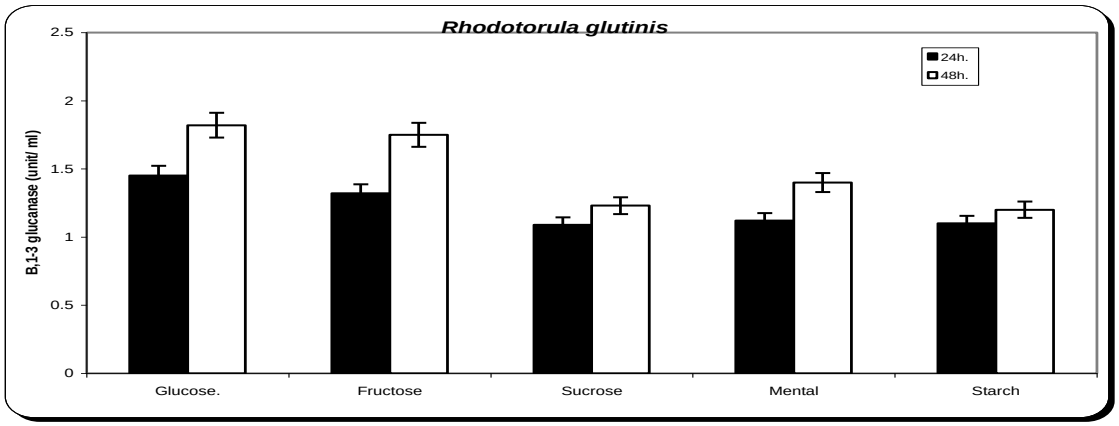

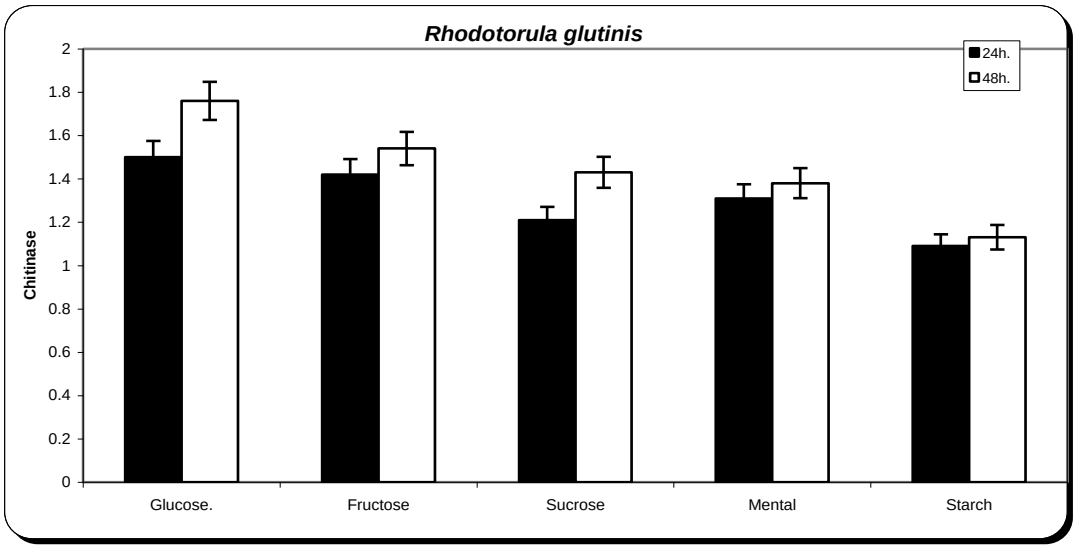

Figure 5: Effect of different carbon sources on the growth, inhibition activity and production of antifungal of *R. glutinis*

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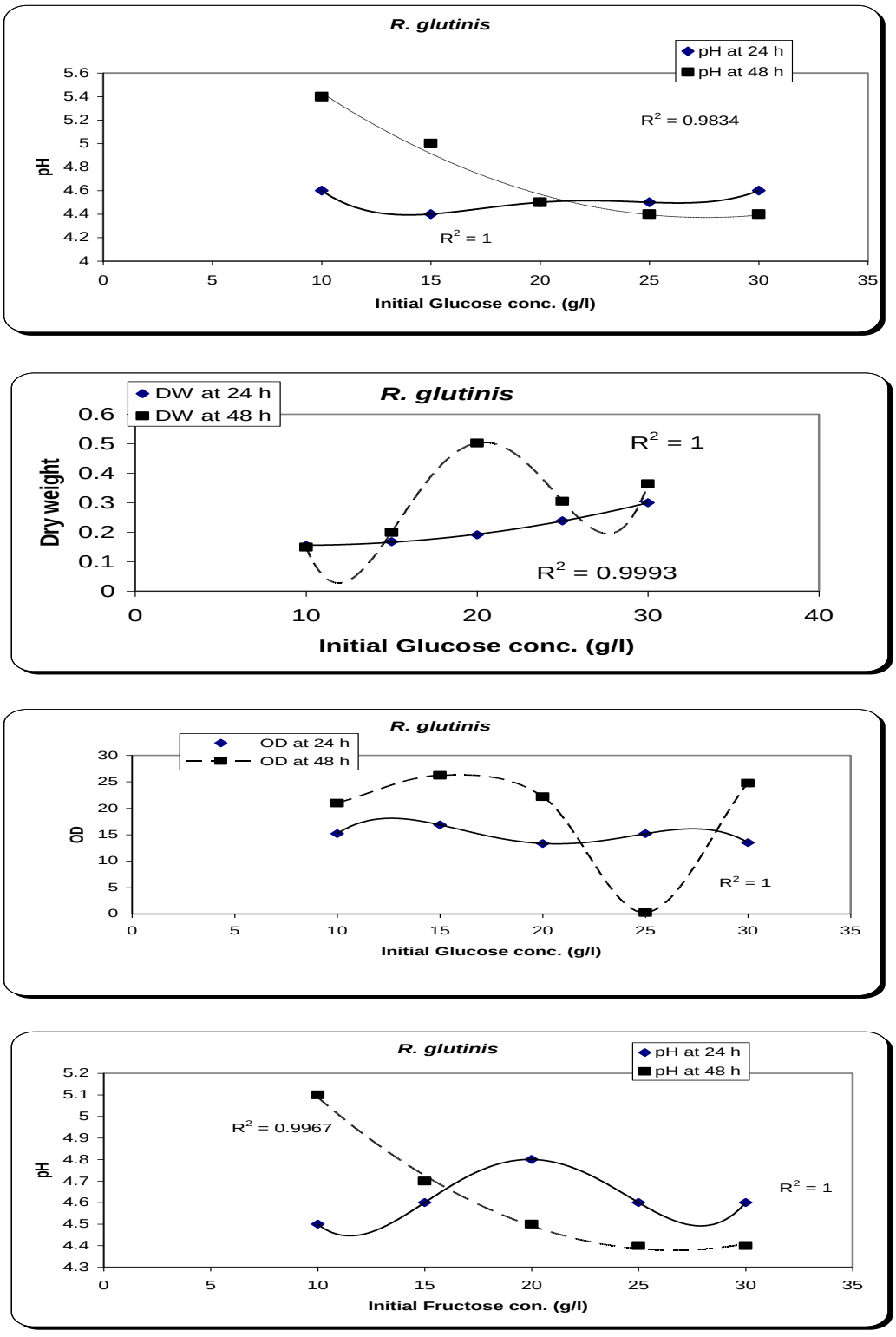

Figure 6: Effect of different carbon concentrations on the growth, inhibition activity and production of antifungal of *R. glutinis*

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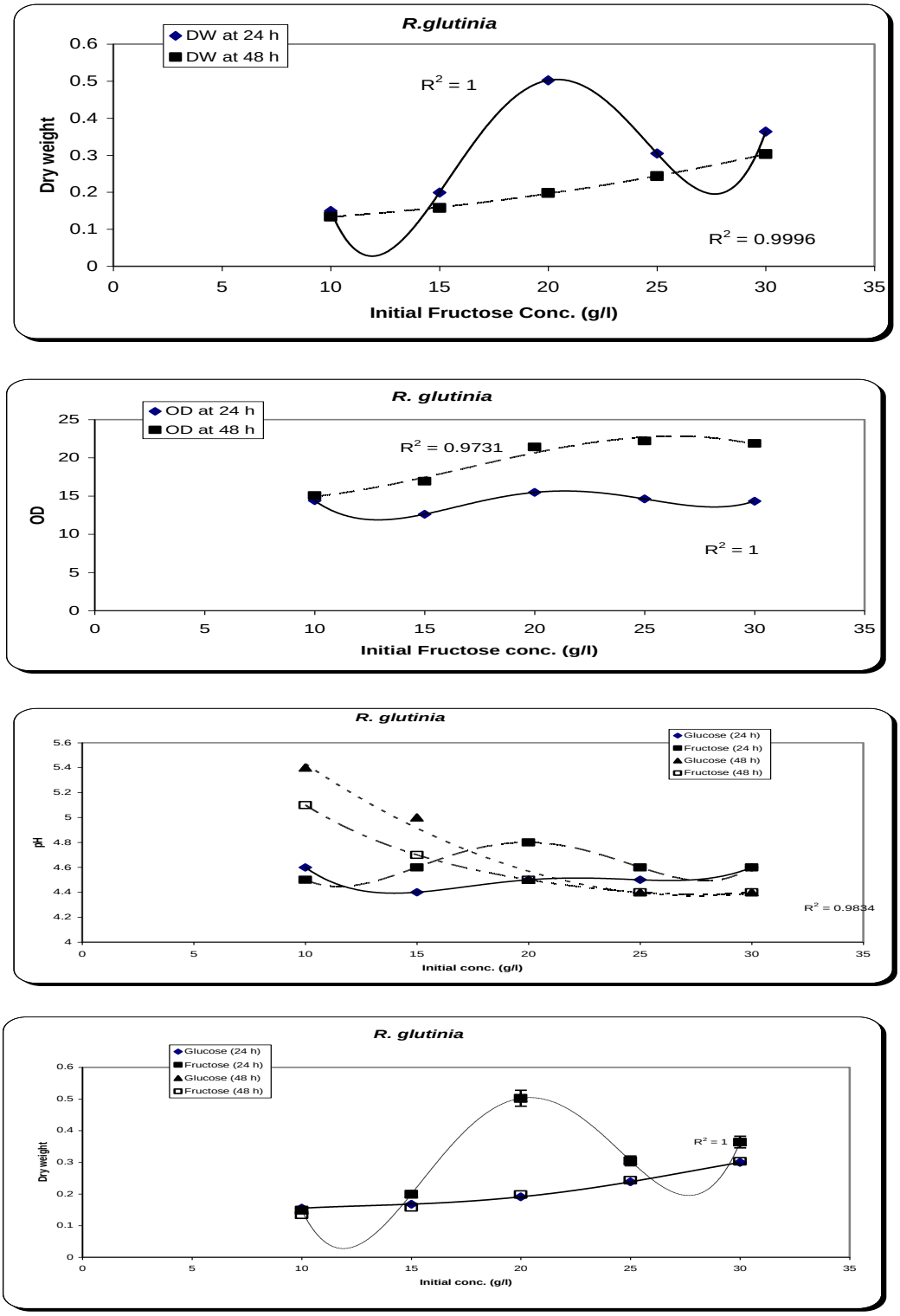

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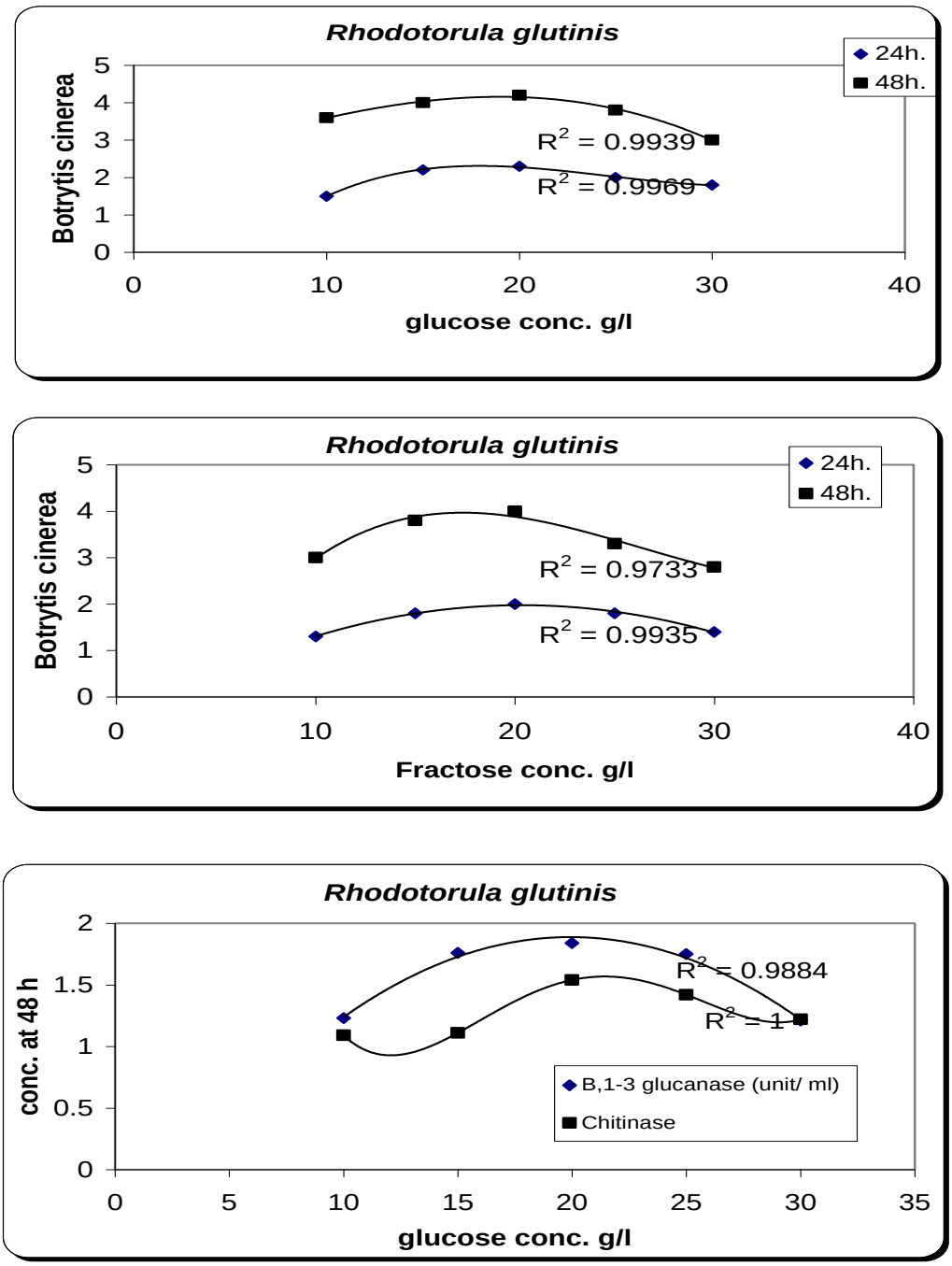
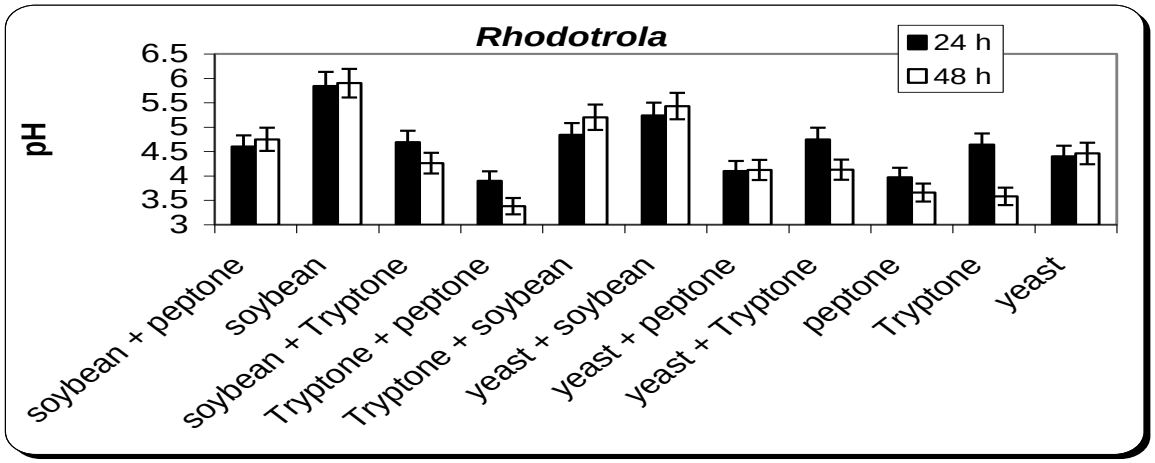
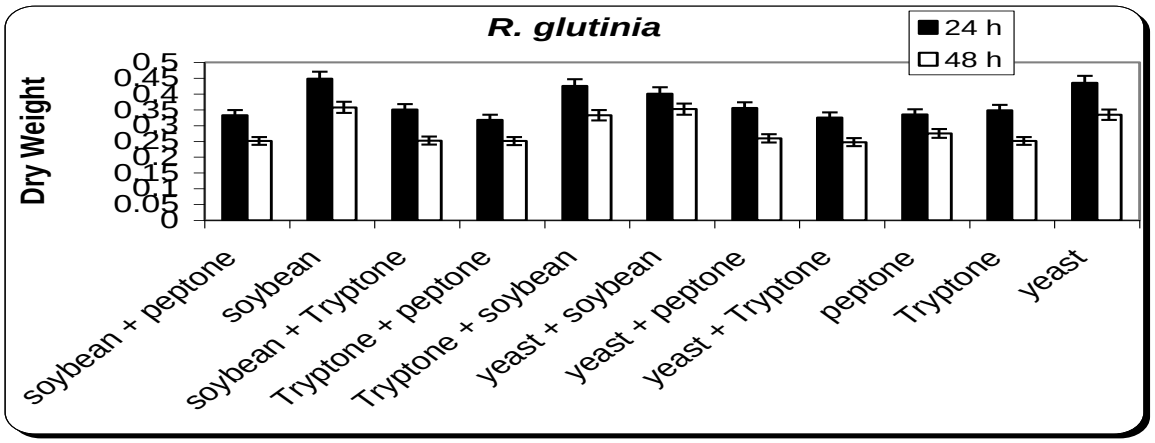


Figure 6: Effect of different carbon concentrations on the growth, inhibition activity and production of antifungal of *R. glutinis*

ISSN: 0975-8585





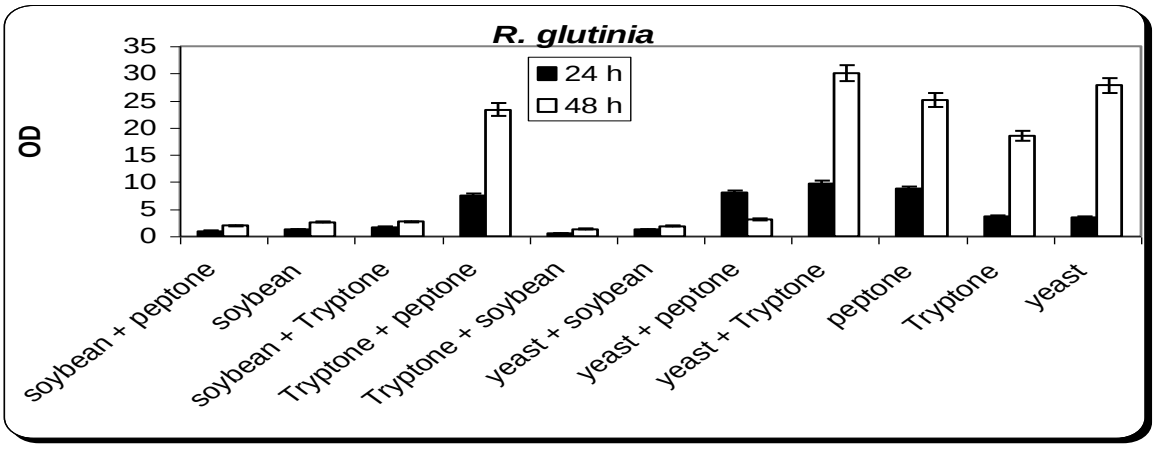
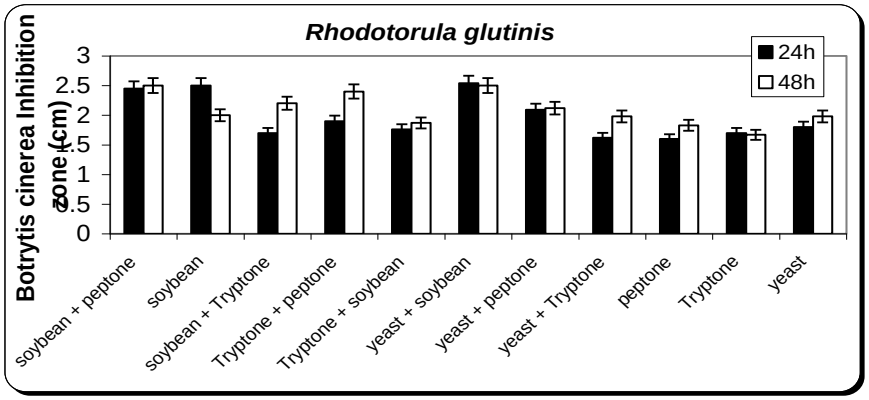


Figure 7: Effect of different nitrogen sources on the growth, inhibition activity and production of antifungal of *R. glutinis*

ISSN: 0975-8585



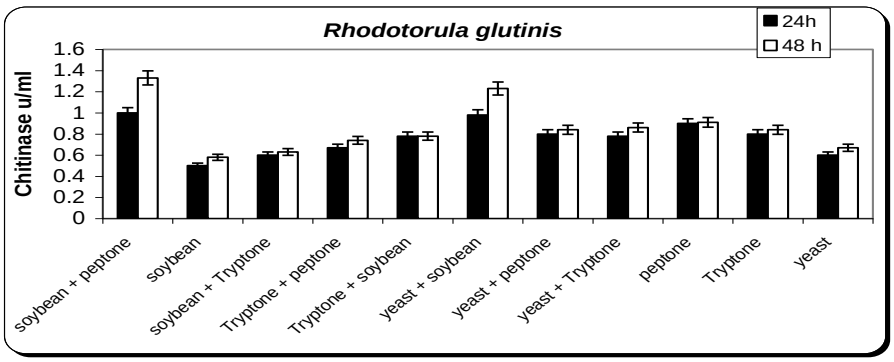


Figure 7: Effect of different nitrogen sources on the growth, inhibition activity and production of antifungal of *R. glutinis*

Fermentation and Formulation

As a part at the fermentation processes development, previous optimum conditions were used for mass production of *Rhodotorula glutinis*. This allowed to continue the development of the liquid fermentation and the scale-up production.

For formulation, liquid formulations used had a longer shelf-life at 4 °C for six months. Glycerol oil was the best formulation used for protect *Rhodotorula glutinis* for 6 months in compared with untreated control (Fig. 8). D-glucose at 1% as sugar sources also gave high effect. Alginate-starch formulation gave high viability during storage until 6 months. The alginate-starch based formulation of *R. glutinis* was excellent until the 6 months of storage, at 4 °C, that showed 97 % viability storage.

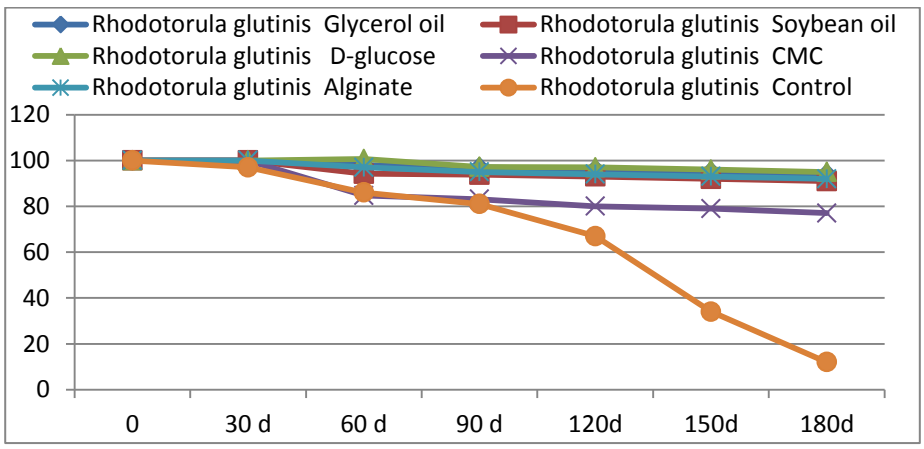


Figure 8: Effect of different formulation on the survival of *R. glutinis* during storage periods

ISSN: 0975-8585

Field tests

Grey mould

The results of foliar application of D-glucose | glycerol oil formulation of *R. glutinis obtained that R. glutinis* was more effective in compared with the chemical fungicide Redomil – plus at concentration of 2 g / l for the control of strawberry gray mould in seasons of 2011 and 2012 (Table 1). Biocontrol efficacy of the *R. glutinis ,* to control *B. cinerea* in non-inoculated strawberry fruits was showed in Table 3. *R. glutinis* treatment reduced the grey mold rot in strawberry fruits. After 7 d of incubation at 18± 1˚C, fruits treated with *R. glutinis* showed better control for *B. cinerea* than those treated with fungicide. in 2011 season and 2012 seasons. The same results also was obtained under artificial condition following storage at 18 ^{0}C for 7 days in both seasons (Table 2) . After 7 days of incubation at 18± 1˚C , treated fruits with *R. glutinis* showed better control of *B. cinerea*. It is evident from the data in Table (3) that applying strawberry with *R. glutinis* significantly improved fruit quality in terms of increasing fruit weight, total soluble solids, and yield compared with fungicide and untreated control in both seasons.

Phomopsis Disease

Liquid formulation of *R. glutinis* was applied under field conditions as large scale to evaluate their effect against *Phomopsis* leaf blight of strawberry plants in seasons of 2011 and 2012. Results in Table (4) and Fig.(9) Indicate that *R. glutinis* reduced the disease severity of leaf blight when applied twice in both seasons. Meanwhile, other treatment showed moderate effect. Preharvest application of liquid formulation of certain microbial isolates provided an effective control of fruit gray mould disease pre and protect fruit during storage under natural and artificial infection of postharvest than the tested fungicide at the recommended levels (Fig. 10).

Effect of liquid formulation of *R. glutinis* on some vegetative characters and yield of strawberry plants under field conditions

The effect of Liquid formulation of *R. glutinis* on some vegetative characters of strawberry plants i.e. number of leaves, fresh and dray weight / plant were determined season of 2011 and confirmed in season 2012 . Results in Table (5 and 6) Indicate that *R. glutinis* had positive effect on fresh and dry weight / plant and yield under field conditions . Meanwhile, other treatments were less effective.

Table 1: Effect of pre harvest treatment with *R. glutinis* on gray mould incidence and rotted tissue part of strawberry fruits under natural infection *in vivo* .

Treatments	Rotted tissue part			Gray mould incidence %	
	2011	2012		2011	2012
R. glutinis	0.0	0.0		0.0	0.0
Fungicide	5.0	1.6		3.0	2.3
Control	20	15.6		10.0	13.3
LSD	0.8	0.6		0.5	0.8

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Table 2: Effect of preharvest treatment with *R. glutinis* on gray mould incidence and rotted tissue part of strawberry fruits under natural and artificial infection of postharvest .

Treatments	Natural infection				Artificial infection			
	Rotted tissue part		Gray mould incidence %		Rotted tissue part		Gray mould incidence %	
	2011	2012	2011	2012	2011	2012	2011	2012
R. glutinis	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fungicide	9.6	8.9	10.3	9.5	32.7	28.5	25.8	28.5
Control	20	100	28	100	100	100	100	100
LSD	3.5	2.7	4.6	3.9	3.6	2.8	4.4	3.8

Table 3: Effect of preharvest treatment with *R. glutinis* on fruit yield in vivo

Treatments	Average fruit diameter (mm)		T.S.S		Weight fruit dry		Yield (tons/ fedan)	
	2011	2012	2011	2012	2011	2012	2011	2012
R. glutinis	52.7	58.8	10.2	9.8	12.3	14.5	13.4	14.5
Fungicide	30.0	38.8	8.8	8.3	10.8	10.6	8.2	9.5
Control	20.0	25.7	8.2	7.4	11.3	10.8	6.7	8.8
LSD	5.6	6.7	0.4	0.6	0.6	0.9	0.7	0.8

Table 4: Effect of *Rhodotorula glutinis* on Phomopsis leaf blight of strawberry plants under field conditions Phomopsis leaf blight scale from 0 -5 according to Louws, (2007) .

Treatments	*Application*	2011 season		2012 season	
		Disease severity	Reduction %	Disease severity	Reduction %
Rhodotorula glutinis	Once	0.8	77.1	0.4	79.0
	Twice	0.3	91.1	0.0	100
Fungicide		1.5	57.0	1.8	61.5
Control	Once	3.5	——	6.9	_
LSD		0.5		0.3	

ISSN: 0975-8585

Table 5: Effect of *Rhodotorula glutinis* on some vegetative characters of strawberry plants under field conditions chlorophyll

Treatments	Application	No. of leaf / plant	Weight (g) / plant			
			Fresh	Increase %	Dray	Increase %
Rhodotorula glutinis	Once	38.5	210	68.0	45.0	50.0
	Twice	39 .7	230	84.0	53.0	76.7
Fungicide		36.1	140		35.0	16.7
Control		30.0	125	—	30.0	—
LSD		0.7	4.3		2.2	

Table 6: Effect of *Rhodotorula glutinis* on strawberry yield under field conditions

Treatments		Yield (tons / feddan)	
		2011	2012
Rhodotorula glutinis	Once	10.5	11.4
	Twice	12.0	14.6
Fungicide		9.0	9.0
Control		6.7	7.5
LSD		0.6	0.8

ISSN: 0975-8585

Figure 9: Effect of *Rhodotorula glutinis* on leaf blight of strawberry plants under field conditions

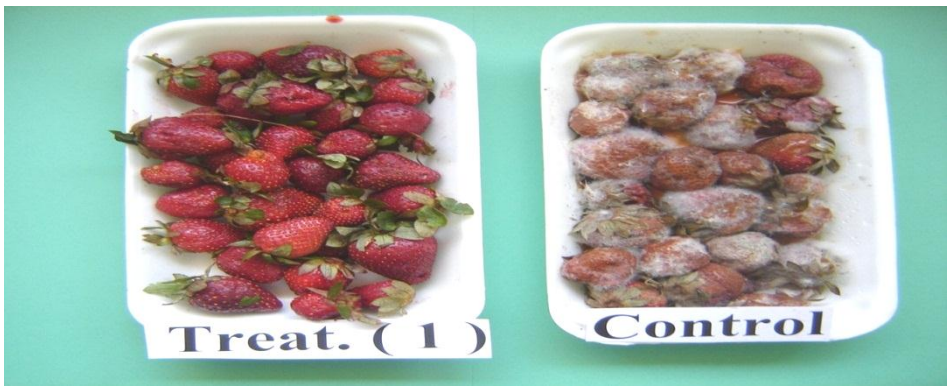

Figure 10: Effect of *Rhodotorula glutinis* on fruit blight of strawberry plants under artificial inoculation of storage conditions

Discussion

Chemical control and use of fungicides are the most effective way of preventing the occurrence of diseases. However, following an increased public health concern and fast development of resistance to novel fungicides by fungi. The use of synthetic chemicals to control plant diseases is to be coming more restricted. The added costs for controlling diseases make losses even more important economically. Biologically based products were identified as possible alternatives to the address these concerns. This has provided an opportunity for the introduction of microbial products as potential alternatives (Haggag, Wafaa, 2008). Antagonistic microorganisms are being studied in depth and considered as an attractive option for the development of microbial-based biofungicides and low doses of chemical fungicides. To develop these isolates as commercial biofungicides, an extensive identification is essential. Therefore, biological, biochemical and molecular technique were conducted with the aim of identification of promising isolates for future development as biological control agents. Mycoparasitism involving lytic enzymes has been already described as the mechanism of action of biological control isolates in the biological control of commercially important plant pathogens (Hamlyn *et al.* 1987) . Reports on glucanases, and the relative importance of any of these systems in the antagonistic process as antifungal agents are well documented (Mukherjee and Sen 2006).

The results of this study provide evidence that, during its growth, the yeast secretes enzymes that are thought to be involved in the degradation of fungal cell walls -b-1,3-glucanase and chitinase, that possibly related to

the biocontrol process. Several microorganisms produce lytic enzymes, which can degrade cell wall of other organisms . Chitinolytic microorganisms are considered to be more effective antagonists of fungal pathogens because of the direct action of chitinase alone (Haggag Wafaa *et al.,* 2012).

The media composition should therefore be further investigated and optimized as the recipe used may have cost implications in higher production regimes. For fermentation process development, recipe development and optimization will form an integral part to ensure and yet economically viable production recipe. The broad spectrum nature of the isolate, even though targeted could also have potential negative outcomes. The impact of product formulation and application on the microbial product has to be properly investigated prior to product registration (Manikandan *et al*., 2010). Members of the genus *Rhodotorula glutinis* have Long been known for their potential to reduce the plant disease caused by fungal pathogens and they have gained considerable importance as potential antagonistic microorganisms. Recently, an antagonistic yeast strain of *Rhodotorula glutinis* have been reported as an effective biocontrol agent (in vivo) against postharvest decay of fruits and vegetables (Yuan *et al*., 2004, Zhang *et al*., 2008, Inas *et al.,* 2010, Haggag, Wafaa ,2013 and Haggag, Wafaa *et al*., 2014). Research on biological control of foliar plant diseases has resulted in the selection of large numbers of micro-organism wit high potential as biological control agents against plant diseases and it could lead to alternatives strategy of plant disease control (Haggag, Wafaa and Nofal, 2006). Our research studies applying bioproducts become more integrated into management strategies (Nofal , and Haggag ,Wafaa, 2006) in protection and curative of plant diseases.

ACKNOWLEDGMENTS

This project was supported financially by the Science and Technology Development Fund (STDF), Egypt, Grant No 216 under title: Development of Bioproducts as Bio-fungicides for Controlling of Major Foliar Diseases of Some Economic Horticultural Crops, from 2009- 2012 ; PI. Wafaa M. Haggag

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