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Enhancement of culture conditions for chitosan production by *Rhizopus nigricans*

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ABSTRACT

The optimal environmental and nutritional conditions for chitosan production by two strains of *R.nigricans* (R6 and R22) were studied. Also, molasses and corn steep liquor were used for maximization chitosan production. Results showed that the highest records of chitosan production by both strains of *R.nigricans* were observed when sucrose (2.5%) and peptone were used as a sole carbon and nitrogen sources, respectively. Concerning the effect of incubation temperatures ,the results emphasized that increasing the incubation temperature led to gradual increase in biomass and chitosan yield up to 28 ˚C for both strains of *R. nigricans*. Moreover, results indicated that the highest values of chitosan production were obtained when *R.nigricans* was incubated at pH 5 for 7 days and decreased thereafter. Results showed that chitosan production by the two *Rhizopus* strains increased with the increasing of corn steep liquor to reach their maximum at 150 g/l. Also, the two *Rhizopus* strains produced higher amounts of chitosan when cane molasses was used as a carbon source rather than beet molasses .Moreover, maximum values of biomass and chitosan were observed when mixture of cane and beet molasses was used in the fermentation medium as a sole carbon source.

Key words: Fungi, *Rhizopus nigricans*, fermentation ,carbon sources, nitrogen sources, environmental conditions, nutritional conditions, molasses and corn steep liquor.

INTRODUCTION

Chitosan is a natural hydrophilic polysaccharide, it is a deacetylated derivative of chitin formed primarily by repeated units of β(1-4)-2-amino-2-deoxy-D-glucose (or D-glucose-amine) and comprising of (1,4)-linked aminodeoxy-β-D-glucan. It is non-toxic, biodegradable,biocompatible and highly polycationic biopolymer**.**The mycelium of several fungi included *Aspergillus niger*, *Rhizopus oryzae* and *Rhizopus* spp have been considered as possible sources of chitin and chitosan.(**Ghonaimy, 2010).**The cell wall of mushrooms could be an important source for chitosan production (**Nitschke *et al,* 2011**). It was used as a component of toothpaste, shampoo and body creams. Also, it was used for lowering serum cholesterol, cell and enzyme immobilization and purification. **(Kim *et al,* 2001).**. Field application of chitosan for inducing resistance against late and early blight diseases of potato and root rot disease of lubin plants **(Abd-Elkareem *et al,* 2001 and Abd-Elkareem *et al,* 2004).**Large amounts of solid wastes are generated during the processing of agro-industrial products, such as cassava and apple. Apple pomace is an important by-product of the apple processing industry. It consists of the press cake remaining after juice extraction from the apples, and contains pulp, seeds and peel **(Streit *et al,* 2009)**.The different chemical compositions of the wastes affected the fungal growth and chitosan production **(Hama *et al,*2006)**. Therefore, this work aimed to investigate the influence of environmental and nutritional conditions for chitosan production by *Rhizopus nigricans*.

MATERIALS AND METHODS

Fungal strains

Rhizopus nigricans (R6 and R22) strains used in this study was isolated and identified in Agric. Botany Department(Microbiology branch) Fac . Agric. Moshtohor ,Benha Univ

Inoculum preparation

Spores of *Rhizopus nigricans* (R6 and R22) were suspended by adding 10ml of sterilized distilled water to the test tube and mixed. Spores suspension was counted using a haemocytometer and adjusted to 10^4 spore / ml.

Selection the best carbon source

Yeast peptone glucose medium **(Chatterjee *et al*, 2005)** was used for *R. nigricans* strains. One hundred ml of prepared medium was supplemented with different carbon sources namely, glucose, fructose, sucrose, maltose, lactose and manitol as a sole carbon source at concentration of 2% for yeast peptone glucose medium. The media were inoculated with the two strains of *R. nigricans* using suitable spore suspension containing 10^4spore / ml. The flasks were incubated at 28°C for 7 days using surface method. Yeast peptone glucose medium for *R. nigricans* strains was modified by replacing the concentrations of sucrose i.e. 2.5, 5, 7.5 and 10%. Flasks containing 100 ml of the best media were inoculated with fungal strain using suitable spore suspension containing (spore / ml. The flasks were incubated at 28°C for 7 days using surface method.

Selection the best nitrogen sources

One hundred ml of medium was dispended in 500 ml Erlenmeyer flasks were supplemented with different nitrogen sources i.e. peptone, urea, ammonium nitrate, ammonium sulphate and potassium nitrate. The media were inoculated using suitable spore suspension containing 10^4 spore /ml. The flasks were incubated at 28°C for 7 days using surface method.

Limitation the best incubation temperature

Flasks containing 100 ml of yeast peptone glucose medium for *Rhizopus* strains were supplemented with the optimum carbon and nitrogen source. Yeast peptone glucose medium was inoculated with *Rhizopus* strains using suitable spore suspension containing 10^4 spore/ml. Inoculated medium was initially adjusted at different temperature values i.e., 22, 24, 26, 28 and 30°C. The flasks were incubated for 7 days using surface method.

Limitation the best incubation period

Modified yeast peptone glucose media which supplemented with optimum carbon and nitrogen sources was inoculated with the two strains of *R. nigricans* using suitable spore suspension containing 10^4 spore/ml .The flasks were incubated for different periods i.e. 3, 5, 7, 9 and 11 days at the optimum temperature.

Selection the best pH value

Yeast peptone glucose medium was initially adjusted at different pH values i.e. 3, 3.5, 4, 4.5 and 5 according to the method described by **Ghonaimy (2010).** The media were inoculated and incubated as described before.

Effect of corn steep liquor as a nitrogen source on chitosan production

One hundred ml of the Yeast peptone glucose medium was dispended in 500 ml Erlenmeyer flasks and supplemented with sucrose as a carbon source. Different amounts of corn steep liquor namely, 25, 50, 75, 100, 125, 150 and 175 g/l were added as a nitrogen source. Flasks containing YPG medium were inoculated with both strains of *R. nigricans* using suitable spore suspension containing 10^4 spore/ml . The flasks were incubated at 28°C for 7 days using surface method.

Effect of molasses as a carbon source on chitosan production

One hundred ml of prepared medium was supplemented with different kinds of molasses as a carbon source namely, cane molasses, beet molasses and mixture of cane and beet molasses (40:60) substituted with equal dose

of sucrose in the synthetic medium and corn steep liquor was added as a nitrogen source (150g/l). YPG medium was initially adjusted at pH 5 and inoculated with *R. nigricans* strains. The flasks were incubated at 28°C for 7 days.

Mycelium harvest

After the incubation period, fungal mycelia were harvested and washed with distilled water, then dried at 60˚C to a constant weight. Chitosan was extracted from dried fungal mycelia according to the method described by **Synowiecki and Al-Khateeb (1997)**

RESULTS AND DISCUSSION

The optimal environmental and nutritional conditions for chitosan production by *R. nigricans* strains:

1-Carbon sources

Data in **Fig.1** showed that *R. nigricans* R22 gave higher records of chitosan production compared to R6, with all different investigated carbon sources. The highest records of chitosan were observed when sucrose was used in fermentation medium as a sole carbon source followed by maltose. The highest amounts of dry chitosan production using sucrose were 137.7 and 406 mg/l for *R. nigricans* R6 and R22, respectively. This result is in agreement with that found by **Kubicek and Rohr (1989)** who stated that sucrose is preferable to glucose as *A. niger* has a potent extracellular mycelium bound invertase that is active at low pH and rapidly hydrolyzes sucrose. Superiority of sucrose over glucose and fructose was demonstrated by **Xu *et al* (1989)**.**Wang *et al* (2008)** studied the effect of different carbon sources on chitosan production from *Absidia coerulea*.They used maltose, sucrose, glucose, fructose, and molasses. They found that sucrose yielded the highest low molecular weight (LMW) chitosan production. It is important to mention that the lowest records of chitosan production by the two strains of *R. nigricans* R6 and R22 (23.6 and 39.5 mg/l, respectively)were obtained when lactose was used in fermentation medium as a sole carbon source.

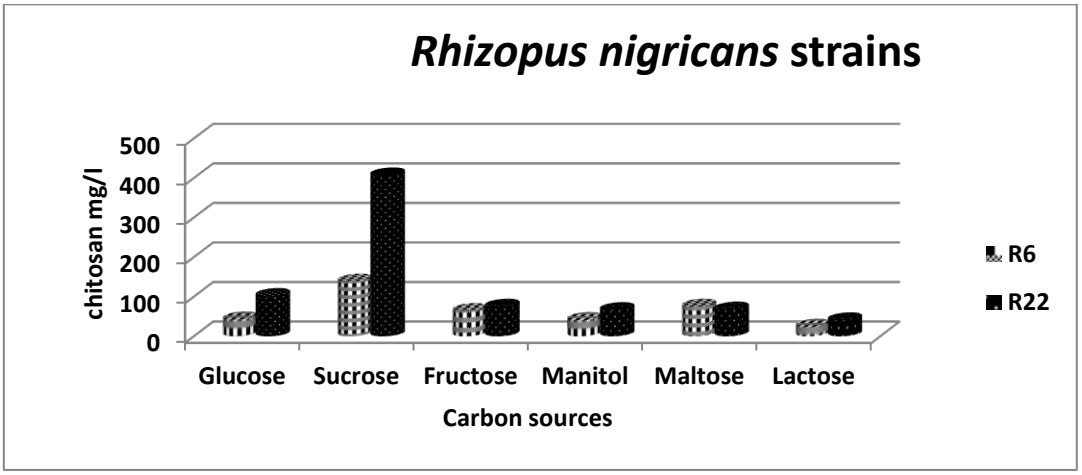

Fig 1. Histogram showing the chitosan production by *R. nigricans* strains using different carbon sources .

2-Sucrose concentrations

Data in **Fig.2** clearly indicated that both *R. nigricans* R6 and R22 produced considerable amounts of chitosan at different concentrations of sucrose. *R. nigricans* R22 gave higher records of chitosan production compared to *R. nigricans* R6 at different concentrations of sucrose.This result is in harmony with **Peksel and Kubicek (2003)** who found that mycelial growth increased and independent of initial sucrose concentration.

Also, data showed that the highest records of chitosan were obtained when sucrose was used in the medium with the percentage of 2.5%. Maximum chitosan was produced with this sucrose concentration, were 139 and 419 mg/l of chitosan yield were obtained by *R. nigricans* R6 and *R. nigricans* R22 strains, respectively. On the other hand, the lowest records of dry chitosan production by the two *Rhizopus* strains were obtained when using 10% sucrose in the medium as a sole carbon source. Minimum amount of chitosan produced with this sucrose concentration were80 and 101 mg/l of chitosan yield were obtained by *R. nigricans* R6 and R22 strains, respectively

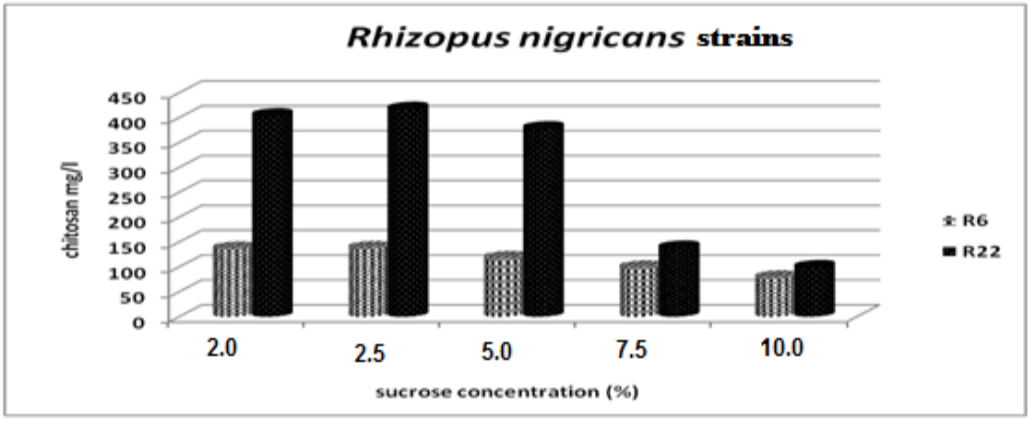

Fig 2. Histogram showing the chitosan production by *R. nigricans* strains using different sucrose concentrations.

3-Nitrogen sources

Data in **Fig.3** emphasized that *R. nigricans* R22 gave higher records of biomass, chitin and chitosan production comparing to *R. nigricans* R6 at different nitrogen sources. The highest records of chitosan production by *R. nigricans* were observed when peptone was used in the fermentation medium as a sole nitrogen source. The amounts of produced dry chitosan were 140 and 428 mg/l for *R. nigricans* R6 and R22 strains, respectively. On the other hand, it is worthily to mention that the lowest records of chitosan production were 66 and 44.8 mg/l for *R. nigricans* R6 and R22 strains, respectively when potassium nitrite was used in the fermentation medium as a sole nitrogen source.

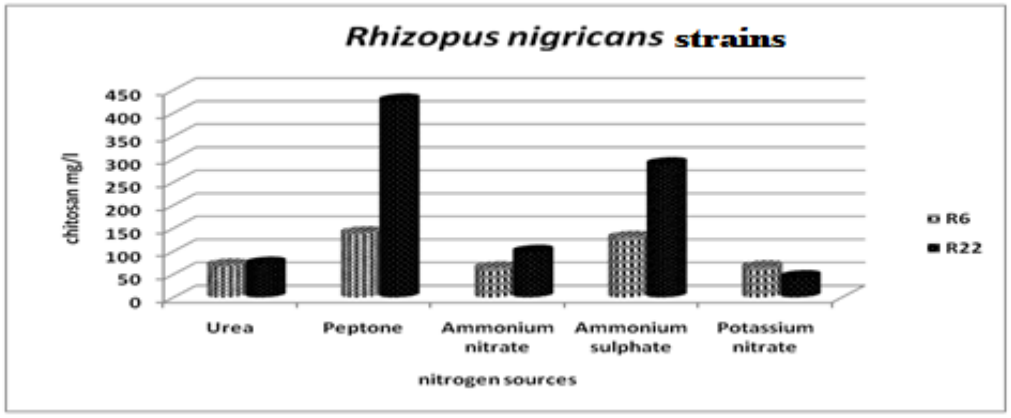

Fig3. Histogram showing the chitosan production by *R. nigricans* strains using different nitrogen sources.

These results are in agreement with those obtained by **Ghonaimy (2010)** who used peptone at the rate of 0.3% to produce the highest yield of chitosan from *Rhizopus nigricans* and *Aspergillus niger*.

4 - Incubation temperature

Data in **Fig .4** clearly indicated that increasing incubation temperature led to gradual increase in biomass and chitosan yield up to 28˚C for both strains of *R. nigricans*. Chitosan yield ranged from 54 to 240 mg/l and from 63 to 427 mg/l for *R. nigricans R* 6 and R22, respectively. From these data, the results clearly showed that *R. nigricans* R22 gave higher values of chitosan compared to *R. nigricans* R6 at all the tested incubation temperature.

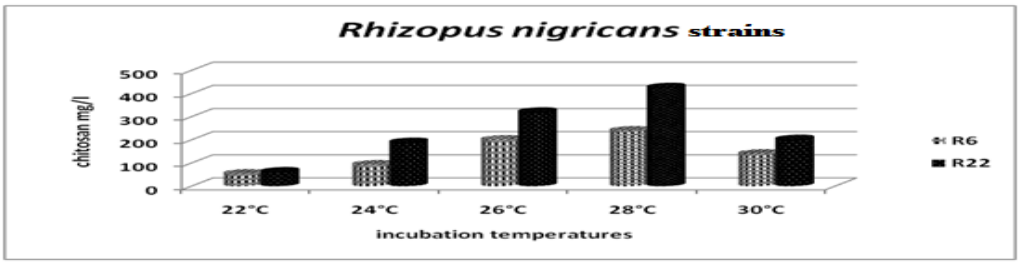

Fig 4: Histogram showing the chitosan production by *R. nigricans* strains different incubation temperatures.

These results are in agreement with those obtained by **Wu *et al*,(2005); Stamford *et al*, (2007) and Ghonaimy, (2010)**who cultured fungal strains such as *Aspergillus niger*, *Rhizopus oryzae*, *Cunninghamella elegans*, *Gongronella butleri* and *Mucor rouxii* at 28°C for chitosan production.

Concerning the effect of incubation temperatures on chitosan production, data notice that the chitosan production amounts by R. nigricans R6 and R22 strains were higher at 26 ˚C incubation temperatures compared to either 24 ^{0}C or 30^{0}C.

5 - Incubation periods

Data in **Fig.5** showed that the values of chitosan production by *R. nigricans* R6, R22 strains were gradually increased with increasing the incubation period to reach their maximum values at 7 days and decreased thereafter.

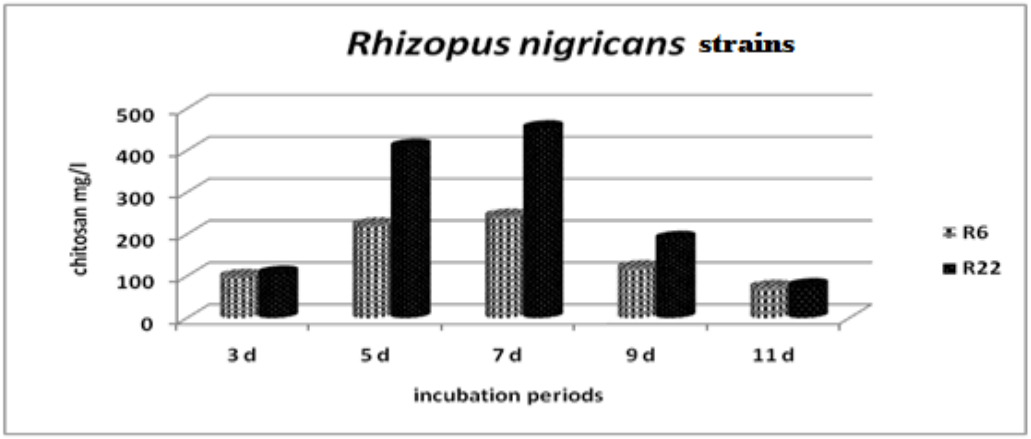

Fig 5: Histogram showing the chitosan production by *R. nigricans* strains using different incubation periods.

The obtained results showed that *R. nigricans* R22 and *R. nigricans* R6 gave the highest values of chitosan when grown for 7 days. On the other hand, the lowest records of chitosan produced by *R. nigricans R* 6 and *R. nigricans* R22 strains were observed at 11 days. The lowest amounts of dry chitosan production were 73 and 77 mg/l for both strains, respectively. These results are in harmony with those obtained by**(Yoshihara *et al.*, 2003)** who found that *Rhizopus oryzae* produced maximum amounts of chitosan in synthetic media at 30°C for 6 days**.** Also, **Ghonaimy (2010)** reported that the growth rate of *Rhizopus nigricans* gradually increased by increasing the incubation period reaching the maximum value at the 7th day and the highest amount of chitosan was 0.78g/l.

6 - pH values

Data in **Fig.6** showed that the highest records of chitosan were obtained at pH 5 for *R. nigricans* R6 and *R. nigricans* R22. The highest amounts of dry weight of chitosan were 401 and 551 mg/l for *Rhizopus sp* R6 and *Rhizopus sp* R22, respectively at pH 5. While, the lowest records of chitosan was recorded at pH 3. were 40 and 89 mg/l for *R. nigricans* R6 and R22 strain, respectively. These results are in agreement with those obtained by **Kleekayai and Suntornsuk (2011**) who studied the effect of different pH values ranged from 3 to 6on chitosan production by *Rhizopus oryzae*. They found that the best chitosan yields were observed at initial pH 5 and 6.

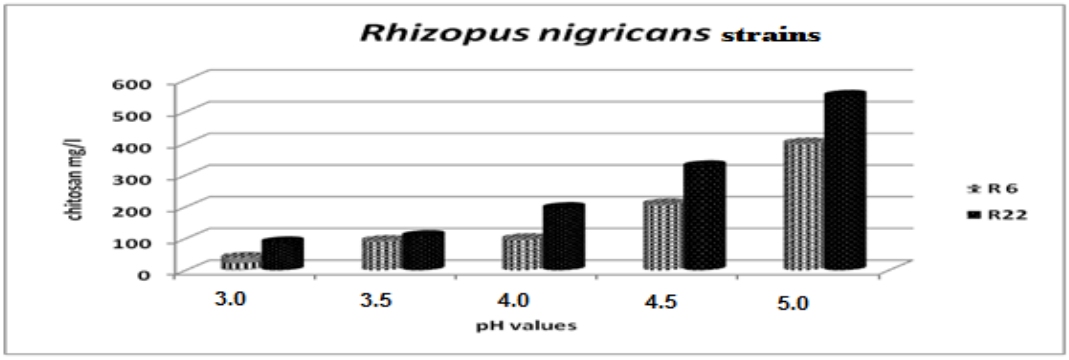

Fig 6. Histogram showing the chitosan production by *R. nigricans* strains using different pH values.

In addition, **Ghonaimy (2010)** studied the effect of different pH values (3 – 6) for chitosan production by *R. nigricans*. He found that the increasing of pH up to 4 led to increase chitosan yield. While, increasing pH up to 6 caused decreasing of chitosan production.

Chitosan production using corn steep liquor as a natural nitrogen source

Data in **Fig.7** showed that the highest amounts of chitosan production at 150 g/l corn steep liquor were 730 and 950 mg/l for *R. nigricans* R6 and R22 strains, respectively. Whereas, the lowest amount of chitosan production which observed at 25 g/l corn steep liquor were 100 and 160 mg/l for *R. nigricans* R6 and R22 strains, respectively. On the other hand the R22 could be utilize the corn steep liquor as a carbon source compared with R6 strain.

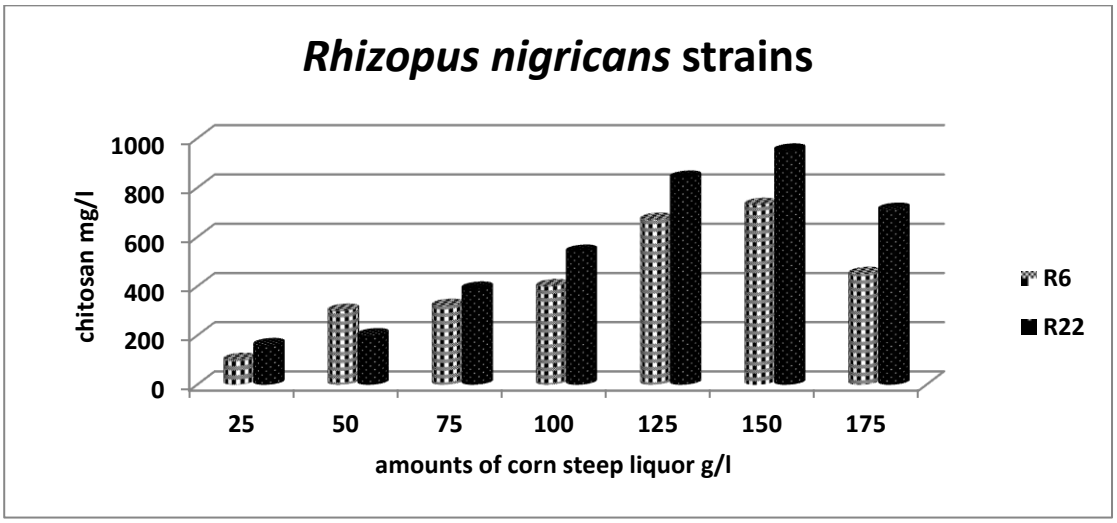

Fig 7: Histogram showing the effect of corn steep liquor on chitosan production by *R.nigricans* (R6 and R22).

These results are in agreement with those obtained by **Cardoso *et al* (2012)**investigated the potential of *Rhizopus arrhizus* for producing chitosan using corn steep liquor and honey as agro industrial nitrogen and carbon sources, they found that the maximum chitosan yield was 29.3mg/g of biomass in culture media comprising corn steep liquor (6%) and honey (13.24%).

Chitosan production using molasses as a natural carbon source

Data in **Fig.8** showed that the two *Rhizopus* strains produced higher amounts of chitosan when cane molasses was used as a carbon source rather than beet molasses. Moreover, maximum values of chitosan were observed when mixture of cane and beet molasses was used in the fermentation medium as a sole carbon source.

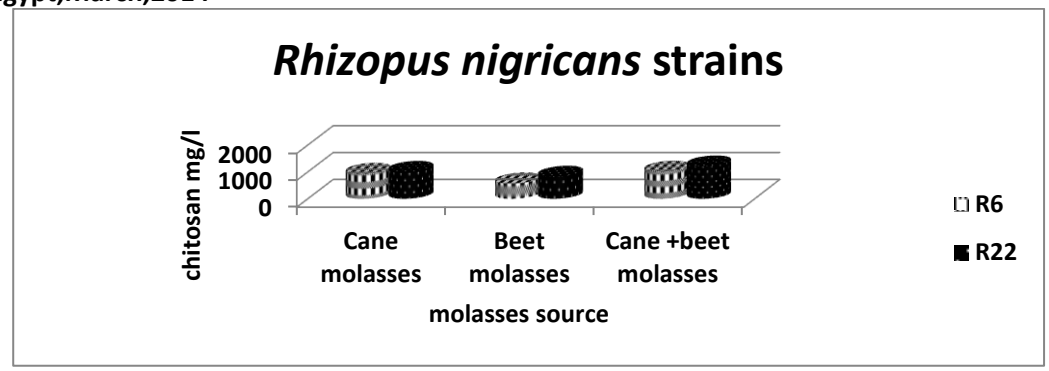

Fig 8: Histogram showing the effect of cane molasses, beet molasses and cane + beet molasses on chitosan production by *R.nigricans*(R6 and R22).

The highest amounts of dry chitosan production were 1010 and 1200 mg/l for *R. nigricans* R6 and R22 strains, respectively. While, the lowest amounts of dry chitosan production were 570 and 820 mg/l for *R. nigricans* R6 and R22 strains, respectively using beet molasses.

CONCLUSION AND RECOMENDATION

In view of the obtained results, it was clearly that the chitosan can be produced by two *R. nigricans* strains .The optimal carbon source was mixture of cane and beet molasses, while, corn steep liquor was the best nitrogen source compared to sucrose and urea, respectively. The optimum of incubation temperature, incubation periods and pH values for chitosan production were 28˚C and for 7 days and 5, respectively.

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تعزيز الظروف المزرعية لإنتاج الشيتوزان بواسطة سلالات من فطر رايزوبس نيجريكانس

راشد عبدالفتاح زغلول ـ حامد السيد أبوعلى ـ طلعت محمد الحسينى– غنيمى عبدالفتاح غنيمى – نهى محمد عشرى

تم إجراء هذه التجربة لدراسة أفضل الظروف المثلي الغذائية والبيئية لإنتاج الشيتوزان بواسطة سلالات من فطر ***Rhizopus nigricans*** ومقارنتها مع بعض المخلفات مثل المولاس وسائل منقوع الذرة. وقد أوضحت النتائج أن أعلي كمية من الشيتوزان كانت عند استخدام السكروز بتركيز 2,5% كمصدر وحيد للكربون . وأعلي كمية من الشيتوزان المنتج بواسطة سلالتي ***R. nigricans***كانت عند استخدام الببتون كمصدر وحيد للنيتروجين.فيما يخص تأثير درجات الحرارة ، أشارت النتائج إلي أنه بزيادة درجة حرارة التحضين يزداد محصول الكتلة الحيوية والشيتوزان لفطر ***R. nigricans*** حتي درجة حرارة 28 ºم لكلا السلالتين. وأيضا أوضحت النتائج أن أعلي إنتاج للكتلة الحيوية والشيتوزان بواسطة سلالتي ***R. nigricans*** يزداد عند التنمية لمدة 7 أيام ثم تنخفض بعد ذلك. كما أن كمية الشيتوزان المنتج بواسطة سلالتي***R. nigricans*** ازدادت مع زيادة pH حتي 5. وعند استخدام بعض المخلفات أوضحت النتائج أن الشيتوزان المنتج بواسطة سلالتي ***R.nigricans*** يزداد بزيادة كمية منقوع الذرة حتي 150 جم/ لتر . علاوة علي ذلك فإن كلا السلالتين أنتجت كميات كبيرة من الشيتوزان عند استخدام مولاس القصب مقارنة بالكميات المنتجة عند استخدام مولاس البنجر ولكن أنتجت أعلي كمية من الشيتوزان عند استخدام خليط بينهما وذلك بالنسبة لكلا من السلالتين.