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Isolation of Scleroglucan-Producing Fungus, *Sclerotium rolfsii* MT-6, from Fermented Squash and the Optimization of Submerged Culture Conditions for Scleroglucan Production

MESUT TASKIN*, SERKAN ERDAL and OZDEN CANLI
Department of Biology, Science Faculty, Ataturk University, 25240 Erzurum, Turkey
Fax: (90)(442)2360948; Tel: (90)(442)2314403; E-mail: mesuttaskin61@hotmail.com

The present study mainly focused on investigating the usability of fermented squashs (*Cucurbita pepo*) for the isolation of new *Sclerotium rolfsii* strains, which is capable of producing scleroglucan in submerged culture. Among 20 *Sclerotium rolfsii* strains isolated, MT-6 was found to be the best strain for both scleroglucan production and mycelial biomass production. Optimal parameters for scleroglucan production by *Sclerotium rolfsii* MT-6 were initial pH of 5, shaking speed of 150 rpm, temperature of 28 ºC, cultivation time of 72 h, sucrose as a carbon source and sodium nitrate and peptone as nitrogen source. Under the optimized submerged culture conditions, maximum productions of scleroglucan and mycelial biomass in shake-flask culture were determined as 15.21 and 17.96 g/L, respectively. The present study showed for the first time that a local isolate of *Sclerotium rolfsii* fungus could produce scleroglucan.

Key Words: *Sclerotium rolfsii* MT-6, Isolation, Scleroglucan, Submerged culture.

INTRODUCTION

The polysaccharides produced by fermentation from a variety of microorganisms are currently used in the food, pharmaceutical and chemical industries. Of the many kinds of microbial polysaccharides, scleroglucan is a non-ionic, water-soluble homopolysaccharide consisting of a linear chain of β-D-(1-3)-glucopyranosyl groups and β-D-(1-6)-glucopyranosyl groups[1]. This polysaccharide shows remarkable properties such as rheological behaviour and stability over a wide range of pH, salinities and temperature, which make it especially attractive for diversity applications. Due to these properties, it finds multifarious applications in the food as a thickener and as gelling agent or stabilizing agent and in pharmaceutical industry as immunomodulatory, antineoplastic and antimicrobial activities that are higher than other glucans and in preparation of controlled release dosage forms[2,3].

Scleroglucan is synthesized extracellularly by species of the genus *Sclerotium*, *i.e.*, *Sclerotium glucanicum*, *Sclerotium rolfsii* and *Sclerotium delphinii*. *Corticium rolfsii* and *Schizophyllum commune* produce other polysaccharides that are structurally similar to scleroglucan. But, the two main species for its production are *Sclerotium glucanicum* and *Sclerotium rolfsii*. *Sclerotium rolfsii* is a heterotrophic filamentous fungus, which is characterized as plant pathogen and parasite. *Sclerotium* species

have brown or black sclerotia (aggregated bodies of hyphae) or light-coloured mycelia and do not sporulate[4]. *Sclerotium rolfsii* survives long term in soil as sclerotia. It is a serious plant pathogen, with a host range of over 200 plant species. This plant pathogen can cause several types of damage, including seedling damping-off, crown and root rot as well as dry rot canker in older plants[5]. Despite of the potential of *Sclerotium rolfsii* to produce scleroglucan, only three strains of this fungus have been reported to be capable of producing scleroglucan with high yield[1,6,7].

Hence, in the present study, we aimed to isolate the scleroglucan-producing new strains of *Sclerotium rolfsii* from fermented squashs (*Cucurbita pepo*) and to optimize some submerged culture conditions for scleroglucan production by the best strain.

EXPERIMENTAL

Isolation of *Sclerotium rolfsii* strains: The isolation of different strains of *Sclerotium rolfsii* fungus was performed from fermented squashs (*Cucurbita pepo*). Portion (less than 5 mm) of the fermented squashs was excised under sterile conditions and immersed in a solution of the solution containing streptomycin (100 μg mL^{-1}) and ampicillin (100 μg mL^{-1}) for 8-10 min. Then, it was transferred into petri dishes containing potato dextrose agar (PDA) supplemented with streptomycin (100 μg mL^{-1}) and ampicillin (100 μg mL^{-1}) and incubated for 7 days at 28 °C. Pure culture was obtained by sub-culturing 4 times. In this way, 20 *Sclerotium rolfsii* strains were isolated. For the identification of *Sclerotium rolfsii* fungus, petri dishes were incubated for 11 days at 28 °C and examined daily for the presence of the characteristic mycelium and sclerotia. The agar media were completely covered by the mycelia of *S. rolfsii* at the 6-8 days after inoculation. The appearance of mycelia was silky white at early stage of growth and became dull in appearance after 10 days of inoculation. The formation of sclerotia by fungus was observed at the edges of the plates after 11-day inoculation. The identifcation of *S. rolfsii* was carried out according to the following identification keys[8,9] using these characteristic properties of mycelia.

Screening of strains: For the selection of the best isolate, 20 strains were separately cultivated in the basal medium containing the following components (%): sucrose 8, sodium nitrate 0.3, yeast extract 0.1, magnesium sulphate 0.025, di-potassium hydrogen phosphate 0.13, citric acid 0.07, potassium chloride 0.05 and ferrous sulphate 0.0005[10]. The pH of media was adjusted to 4.5 by using 0.1 M HCl and NaOH. The strains were initially grown on petri-dishes containing potato dextrose agar (PDA) for 7 days at 28 °C. Then, actively growing mycelia of each strain was inoculated into 250 mL conical flask containing 100 mL of the basal medium by punching out one cm of PDA with a sterilized self-designed cutter. The flasks were incubated at 28 °C on a rotary shaker incubator at 100 rpm for 48 h. Maximum scleroglucan production was achieved with the strain MT-6. Hence, following experiments were performed using this strain.

Scleroglucan production with *Sclerotium rolfsii* MT-6 in submerged culture: To produce scleroglucan with *Sclerotium rolfsii* MT-6, we first performed the experiments regarding the optimization of initial pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5), shaking speed (100, 150 and 200 rpm) and temperature (22, 25, 28, 31 and 34 ºC) for 48 h. Then, effect of cultivation time (24, 48, 72, 96 and 120 h) on scleroglucan and mycelial biomass production was investigated. The influence of carbon sources was studied in the basal medium containing various carbon sources, *i.e.,* glucose, sucrose, maltose, fructose and lactose, where each carbon source was added to the medium at 8 % concentration. To investigate the effect of different nitrogen sources on scleroglucan production yeast extract was replaced with other organic nitrogen sources such as peptone, casein and beef extract at 0.1 % concentration, whereas the sodium nitrate (inorganic nitrogen source) was replaced with potassium nitrate, ammonium chloride, ammonium sulphate, urea at 0.3 % concentration. In the case of screening experiments, optimization experiments of these parameters were performed in 250 mL conical flasks containing 100 mL of the basal medium.

Analytical methods: The isolation of scleroglucan from fermented medium was carried out according to procedure described by Farina *et al.*[7], Scleroglucan was recovered by filtration under vacuum and dried at 105 °C until its weight became constant. Similarly, the pellets or mycelia for the determination of biomass concentration were washed with distilled water and dried at 105 °C to constant weight.

Statistical analysis: Each experiment was repeated at least three times 2 replicates. Each experiment was repeated at least three times 2 replicates. Statistical analysis was performed using one way analysis of variance (ANOVA). $p = 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Isolation and screening of scleroglucan-producing strains of *Sclerotium rolfsii*: S*clerotium rolfsii* is a scleroglucan-producing filamentous fungus, which is characterized as plant pathogen and parasite[4]. However, it was reported that only three strains of this fungus had ability to produce scleroglucan in submerged culture[1,6,7]. In this context, it is believed that there is an urgent need to the exploration of new *Sclerotium rolfsii* strains, which have the potential to produce scleroglucan. The present experiments demonstrated that *Sclerotium rolfsii* fungus could be isolated from 20 of fermented 25 squashs (*Cucurbita pepo*). This meant that fermented squash was a good source for the isolation of scleroglucan-producing fungus *Sclerotium rolfsii*. On the other hand, the screening of isolated microorganisms is accepted as very important aspect to obtain microorganisms with high ability or to improve the productivity of some known microbial products. Therefore, in the second step, we performed the screening experiments in order to establish the best strain. The second step experiments indicated that although all of twenty strains

had potential to grow and produce scleroglucan in the basal medium (screening medium), MT-6 was found to be the best productive strain in terms of both mycelial biomass production (12.63 g/L) and scleroglucan production (4.02 g/L). Hence, the following experiments for scleroglucan production were carried out with *Sclerotium rolfsii* MT-6.

Effects of temperature, shaking speed and initial pH on scleroglucan and mycelial biomass production: Effects of initial pH, temperature, shaking speed and on scleroglucan and mycelial biomass production were investigated in in the basal medium. The results regarding the optimization of initial pH, temperature and shaking speed were summarized in Table-1. An initial pH of 5 supported maximum production of 4.56 g/L of scleroglucan, whereas maximum biomass production (12.63 g/L) was obtained at a pH 4.5. This finding was not in agreement with those reported in the previous studies[10,11]. The previous studies reported that optimum pHs for biomass production and scleroglucan production were 3.5 and 4.5, respectively. Scleroglucan production was maximum (6.25 g/L) at a shaking speed of 150 rpm, whereas mycelial biomass production was maximum (15.11 g/L) at a shaking speed of 200 rpm. Among the temperatures tested, a temperature of 28 ºC resulted in the

TABLE-1
EFFECTS OF pH, SHAKING SPEED AND TEMPERATURE ON MYCELIAL GROWTH AND SCLEROGLUCAN PRODUCTION

Culture parameters / Initial pH	Mycelial biomass (g/L)	Scleroglucan (g/L)
4.0	10.11 ± 0.055	2.88 ± 0.015
4.5	12.63 ± 0.061	4.02 ± 0.014
5.0	11.52 ± 0.045	4.56 ± 0.029
5.5	10.15 ± 0.047	3.18 ± 0.025
6.0	9.34 ± 0.045	2.61 ± 0.020
6.5	8.45 ± 0.056	2.03 ± 0.015
Shaking speed (rpm)		
100	11.52 ± 0.045	4.56 ± 0.029
150	14.03 ± 0.064	6.25 ± 0.019
200	15.11 ± 0.053	5.41 ± 0.014
Temperature (°C)		
22	12.33 ± 0.033	3.51 ± 0.009
25	13.38 ± 0.041	4.71 ± 0.011
28	14.03 ± 0.064	6.25 ± 0.019
31	15.22 ± 0.066	5.58 ± 0.023
34	13.11 ± 0.039	3.16 ± 0.012

Optimization of initial pH: shaking speed = 100 rpm, temperature = 28 ºC and cultivation time = 48 h. Optimization of shaking speed: initial pH = 5.0, temperature = 28 °C and cultivation time = 48 h. Optimization of temperature: initial pH = 5.0, shaking speed = 150 rpm and cultivation time = 48 h (carbon source = sucrose (8 %), organic nitrogen source = 0.1 % yeast extract and inorganic nitrogen source = 0.3 % sodium nitrate). All values are mean of three times 2 replicates ± SD.

maximum scleroglucan production (6.25 g/L), whereas maximum biomass production (15.22 g/L) was achieved with a temperature of 31 ºC.

These results meant that the optimum values of initial pH, temperature and shaking speed were different for the mycelial growth and the biosynthesis of scleroglucan in *Sclerotium rolfsii* MT-6. However, the following experiments were performed at the fermentation parameters (pH = 5, temperature = 28 ºC, shaking speed = 150 rpm and cultivation time = 72 h) which were determined as optimal for scleroglucan production, since the present study focused on mainly scleroglucan production rather than mycelial biomass production.

Effect of carbon and nitrogen sources on scleroglucan production and mycelial growth: Carbohydrates are a major component of the cytoskeleton and an important nutritional requirement for growth and development of fungi. Utilization of carbon sources varies between fungal species, even if same fungal species also needs different carbon source for specific metabolite production[12]. Therefore, selection of the carbon source in the fermentation studies is critically important. The present study displayed that the mycelial growth of the *Sclerotium rolfsii* MT-6 occurred in a varieties of carbon sources; however, production of mycelia and scleroglucan were quite distinct (Table-2). Among the carbon sources examined, sucrose resulted in the highest scleroglucan production (6.25 g/L). Following this, high scleroglucan production was achieved with glucose. In contrast to scleroglucan production, the maximum production (15.14 g/L) of mycelial biomass was achieved with maltose. These results elucidated that each carbon source was independently responsible in mycelial growth and scleroglucan production. The similar results were also observed in submerged cultivations of *Sclerotium rolfsii* MTCC 2156 and *S. rolfsii* ATCC 201126[1,10].

The data presented in Table-2 show the effect of different organic and inorganic nitrogen sources on scleroglucan and mycelial biomass production. Among the organic nitrogen sources tested, peptone resulted in both maximum scleroglucan production (6.56 g/L) and maximum mycelial biomass production (14.42 g/L). This result was not agreed with that reported by some investigators[1,10]. They claimed that yeast extract was the best organic nitrogen source for maximum productions of mycelial biomass and scleroglucan. As for the effect of inorganic nitrogen sources, sodium nitrate was found to be the best for the maximum productions of scleroglucan and mycelial biomass. This result was in good agreement with that reported by the same investigators[1,10].

Effects of cultivation time on scleroglucan production and mycelial growth: The results presented in Fig. 1 show that scleroglucan poduction occurred simultaneously with growth for the first 48 h of cultivation. Biomass exhibited slight increases after 48 h and reached to maximum (17.96 g/L) at 108 h, while scleroglucan exhibited significant increase after 48 h and reached to maximum (15.21 g/L) at 72 h.

TABLE-2
EFFECTS OF CARBON AND NITROGEN SOURCES ON MYCELIAL GROWTH AND SCLEROGLUCAN PRODUCTION

Medium component	Mycelial biomass (g/L)	Scleroglucan (g/L)
Carbon source (8 %)		
Sucrose	14.03 ± 0.064	6.25 ± 0.019
Glucose	13.52 ± 0.071	5.46 ± 0.011
Fructose	12.05 ± 0.032	4.05 ± 0.016
Maltose	15.14 ± 0.059	4.21 ± 0.022
Lactose	8.45 ± 0.021	2.61 ± 0.008
Organic nitrogen source (0.1 %)		
Yeast extract	14.03 ± 0.064	6.25 ± 0.019
Peptone	14.42 ± 0.079	6.56 ± 0.012
Casein	13.11 ± 0.050	5.85 ± 0.033
Beef extract	12.66 ± 0.041	5.56 ± 0.024
Inorganic nitrogen source (0.3 %)		
Sodium nitrate	14.42 ± 0.079	6.56 ± 0.012
Potassium nitrate	13.08 ± 0.024	5.61 ± 0.020
Ammonium chloride	12.42 ± 0.036	5.28 ± 0.012
Ammonium sulphate	11.33 ± 0.019	4.78 ± 0.019
Urea	11.26 ± 0.023	4.66 ± 0.013

Optimization of carbon source: organic nitrogen source = yeast extract (0.1 %) and inorganic nitrogen source = sodium nitrate (0.3 %). Optimization of organic nitrogen source: carbon source = sucrose (8 %) and inorganic nitrogen source = sodium nitrate (0.3 %). Optimization of inorganic nitrogen source: carbon source = sucrose (8 %) and organic nitrogen source = 0.1 % peptone extract (initial pH = 5.0, shaking speed = 150 rpm, temperature = 28 °C and cultivation time = 48 h). All values are mean of three times 2 replicates ± SD.

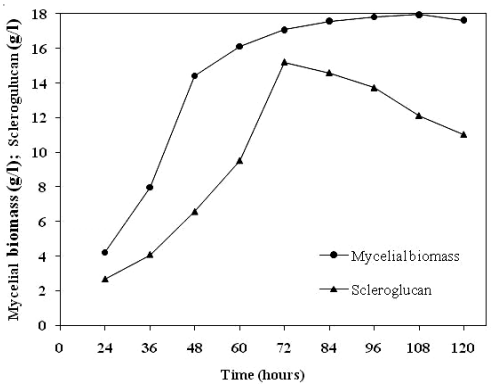

Fig. 1. Effect of cultivation time on mycelial biomass and scleroglucan production by *Sclerotium rolfsii* MT-6. Fermentation parameters: initial pH = 5, shaking speed = 150 rpm, temperature = 28 °C, carbon source = sucrose (8 %), organic nitrogen source = 0.1 % peptone and inorganic nitrogen source = 0.3 % sodium nitrate

There were significant decreases in scleroglucan concentration after 72 h of cultivation. This could be due to the production of glucanases, which degrades the polysaccharide[10,13].

The present results revealed that manipulation of nutritional components and environmental conditions allowed to significantly increase scleroglucan production by *S. rolfsii* MT-6. Maximum productions of scleroglucan and mycelial biomass were found to be (15.21 g/L) and (17.96) under the optimized submerged culture conditions, respectively. The amount of scleroglucan produced in the present study was considerably near to that reported by Survase *et al.*[10]. They reported that *Sclerotium rolfsii* MTCC 2156 produced scleroglucan of 16.58 g/L in the production medium with the same composition. This result meant that *S. rolfsii* MT-6 was a promising strain in industrial fermentation studies regarding scleroglucan production.

Conclusion

It is well known that there has been a growing interest in isolation of microorganisms from various sources for production of industrially valuable microbial products. In this context, the present study elucidated that fermented squashs (*Cucurbita pepo*) was a good source for isolation of scleroglucan-producing strains of *Sclerotium rolfsii* fungus. On the other hand, in developing an optimal process for commercial production of microbial products, optimization of environmental conditions and nutritional components is considered as a major aspect for improvement. The present study also demonstrated the optimization of these parameters significantly improved scleroglucan production. From above results, it is believed that the present study can make significant contributions to studies regarding industrial fermentation and food microbiology.

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