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Detection of the compounds produced from *Rhizopus oryzae*

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Abstract

Rhizopus oryzae is classified a one of the Mucorales which belong to the zygomycota. This microorganism is a filamentous fungus can produce various substances such as ethanol, fumaric acid, lactic acid, ragi, and temph. *R. oryzae* has been occupied important role in the producing benefit metabolites such as organic acids. Enzymes. and antimicrobial compounds. In the current study, potato dextrose agar (PDA) and malt extract agar (MEA) were used for growing *R. oryzae* that the fungus was identified depending on the morphological parameters and conventional PCR. The fungus was cultured in two synthetic media were corn steep liquor (SCL) and conocarpus leaves (CL) at 25°C. *R. oryzae* produced abundant growth in both media and the growth was greyish white. GC-MA analysis appeared several different compounds in the crude extracts of *R. oryzae* using these fermenting synthetic media; however, some compounds were reported to be similar in their structures. The similar compounds were (pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-), (1-nonadecene), (pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl),) methyl stearate), (hexanedioic acid, bis(2-ethylhexyl) ester), (bis(2-ethylhexyl) phthalate), and (1,4-benzenedicarboxylic acid, (bis (2-ethylhexyl) ester). This study aimed to evaluate CL medium in comparison with CSL one for detecting the natural compounds produced from *R. oryzae*.

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Introduction

Rhizopus oryzae is classified as one of the Mucorales which belong to the Zygomycota. This microorganism is a filamentous fungus that can produce various substances such as ethanol, fumaric acid, lactic acid, lipase ragi, enzymes, and temph (1-7). *R. oryzae* is essential in producing beneficial metabolites such as organic acids, enzymes. and antimicrobial compounds. Its presence as either pathogenic or saprophytic forms represents a source of the producing metabolites such as organic acids, polymers, enzymes, esters etc. Examples of enzymes are protease, amylase, cellulose, tannase, hemicellulose, phytase, and lipase (8-11). Moreover, this fungus is traditionally used to give other beneficial products such as, volatile materials, bio alcohols, and recipes (8,12). *R. oryzae* possesses a role in the probiotic aspect that participates in the digestion of nutrients and preventing pathogens especially in the poultry industry (13). In industrial mycology, the fungus was tested to activate antioxidant compounds in the food preparation such as the cake of pumpkin oil (14). Compositions of the media and their manipulation show success in the discovering natural products. These compositions lead to fungal secondary metabolites, if any nutritional variations of the medium can affect the on a production of the metabolites (15,16). Related to the media, *R. oryzae* can increase produced lipase and pectinase when growing in a medium containing carbon source supplemented with olive oil. This showed that the carbon source with olive oil had a remarkable effect on the fungal growth and enzyme activity. As well as pellet morphology and other growth kinetics were reported due to composition of the medium (17-23). Other researchers

reported the effects of the remains obtained from the agro-industrial products on the producing fumaric acid by *R. oryzae*. They used these residues as a substrate for giving that acid (24-26). Concerning agricultural residues investigated trash of the sugar can to improve the capability of *R. oryzae* for producing fumaric acid and their results concluded that this fungus is a potent source of the acid and the rash represents a suitable substrate and strategy for obtaining the fumaric acid (27).

This study aimed to evaluate the CL medium in compared to CSL one for detecting the natural compounds produced by *R. oryzae*.

Materials and methods

Ethical approval

According to a protocol approved by the Bioethics Committee for Animal numbered UM.VET.2021.069.

Growth and identification of *Rhizopus oryzae*

Rhizopus oryzae was isolated from poultry food, diagnosed using conventional and molecular methods and sub- cultured on potato dextrose agar (PDA) and malt extract agar (MEA) at 25°C, 5 days. The fungus was identified depending on the morphological characteristics including macroscopic and microscopic properties (28) and molecular identification of fungal, primer had been chosen alignment specific feel corresponding to the sequences MucL1 (5`-TGATCTACGTGACATATTCT-3`) MR1 (5`-AGTAGTTTGTCTTCGGKCAA-3`) 836 bp size (29). Fungal genomic DNA was extracted from fungi and prepared from PDA culture using ABIO pure DNA extraction kit (30). Nano-drop spectrophotometer was used to check the concentration of the extracted DNA following the formula: 1OD260=50ng, purity= 260/28. PCR amplification and PCR master mix reaction used to be organized with the aid of the GoTag Green Master Mix kit protocol. PCR computer was as noted in table 1, the PCR computer was set up, for 30 cycles. PCR products have been visualized via agarose gel stained of ethidium bromide dye (Biometra, Germany).

Table 1: PCR program setting for fungal isolates

Step	Temp.	Time	Cycles
Initial denaturation	95ºC	5 min	1
Denaturation	95ºC	30 sec	30
Annealing	60ºC	30 sec	
Extension	72ºC	30 sec	
Final extension	72ºC	7 min	
Hold	10ºC	10 min	1

The fermentation processes

Two synthetic media were used for the fermentation process, the first medium was steep corn liquor (CSL) supplemented with other substances. This medium composed (per liter) of 5 ml, 50, 20, 2, 0.87, 0.5, 0.5, and 20 ml of CSL powder, glucose, peptone, yeast extract, potassium dihydrogen phosphate, magnesium sulfate, calcium chloride, and trace metal solution, respectively. The trace metal solution was prepared by 0.01, 0.1, 0.015, 0.161, 0.01, and 0.01 g of cobalt nitrate, iron (II) sulfate, copper sulfate, zinc sulfate, manganese sulfate, and ammonium molybdate, respectively. The pH of the CSL medium was adjusted at 5.5 (15). Second medium CL, modified, was prepared with the same components except powder of CSL was replaced by 5 ml of the *Conocarpus* species (Figure 1) leaves filtrate was obtained from washed leaves with tap water and then distilled water. The washed leaves were left at room temperature until getting their dryness, cut and pulverized by using a small mill to be powder. 5 g of the powder was dissolved in 1 liter of distilled water and filtrated through filter paper. However, the pH of the CL medium was also adjusted to 5.5. Four glass flasks were prepared and each flask (1 liter capacity) contained 250 ml of each medium two flasks contained CSL volumes and two had the same volumes as the CL medium. Two flasks of both media were used as control while other flaks were fermented in which each flask was inoculated with two PDA discs (6 mm in diameter for each disc) of 10 days old *R. oryzae* colony. All flasks including control ones were incubated at 25°C for 10 days.

Figure 1: Tree of *Conocarpus* species.

Extraction and detection of fungal crude extract

After incubation, filter paper separated the fungal filtrate from mycelia. The filtrate was mixed with the same volume of the chloroform using a separator funnel. Chloroform resulted in two organic layers where the lower layer was selected and left at 25°C for evaporation. The residue of the lower layer was dissolved with 10 ml of absolute methanol and filtrated. Methanol filtrate was subjected to a GC-MS device. An Agilent Technologies carried out GC-MS analysis, 7890B GC method coupled to an Agilent Technologies 5977A MSD with EI sign detector, with HP-5 ms 5% phenyl, 95% methyl siloxane (30). The oven

temperature used to be set at 40°C for 5 minutes, then raised to 10°C/min. to 300°C for 20 minutes. Helium issuer and gas drift rate used to be as soon as 1 ml/min. and purge glide of 3 ml min. The injection mode was once as soon as pulsed splitless with an injection temperature of 290°C, and the injection pattern extent used to be 1 µm. the mass spectrometer used an ion supply and 230°C, with a scan velocity of 1562 (N2) and a mass vary used to be 44-750 m/z. Data used to be run via the NIST 2014, library data base as a greater device to affirm the identification of the fungal compounds.

Results

Identification of *Rhizopus oryzae*

Rhizopus oryzae appeared rapid showed aerial growth during 3-5 days and filled the Petri dish. On PDA, the colony was wool like and whitish grey and the formation of a blackish brown collar around the growth when the incubation period (10 days) increased. On MEA, the growth of this fungus resulted in a greyish white and the growth collar was less brown than the growth on the PDA. Microscopically, lactophenol cotton blue stained slide revealed non-septate hyphae, sporangia, sporangiophores, rhizoides, and ellipsoidal conidia, they were shown individually and in clusters concerning molecular identification, PCR detected base pairs of the fungus nucleotides in 836 bp of the MucL (Figure 2). Concerning morphology, *R. oryzae* produced abundant greyish growth in both CSL and CL media.

GC-MS analysis of the crude extracts

GC-MS analysis appeared many different compounds (Tables 2 and 3; Figure 3) that crude extracts of *R. oryzae* contain two fermenting synthetic media; however, some compounds were reported to be similar. The similar compounds were (pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-), (1-nonadecene), (pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, (methyl stearate), hexanedioic acid, bis(2-ethylhexyl) ester), (bis(2-ethylhexyl) phthalate), and (1,4benzenedicarboxylic acid, bis (2-ethylhexyl) ester).

Table 2: GC-MS analysis of the compounds produced by *R. oryzae* using CL medium

Peaks	RT	Compound
1	10.503	Triethanolamine
2	13.527	Cyclopropane, 1-methyl-2-octyl-
3	14.774	4-Undecene, (E)-
4	14.965	Hexadecane, 1-chloro-
5	15.747	5-Tetradecene, (E)-
6	16.359	Cyclododecane
7	16.463	Pentadecane
8	18.852	2-Tetradecene, (E)-
9	18.933	Hexadecane
10	20.261	3-Pyrrolidin-2-yl-propionic acid
11	20.526	3-Pyrrolidin-2-yl-propionic acid
12	20.748	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
13	21.08	1-Nonadecene
14	21.397	1,2-Cyclopentanedione, 3,3,5,5-tetramethyl-
15	21.655	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
16	22.444	Hexadecanoic acid, methyl ester
17	22.555	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
18	22.673	1,2-Cyclopentanedione, 3,3,5,5-tetramethyl-
19	23.100	3-Eicosene, (E)-
20	23.403	Cyclobutane, 2-hexyl-1,1,4-trimethyl-, cis-
21	23.491	Cyclopentanone, 2-(phenylthio)-
22	24.354	Methyl stearate
23	24.767	l-Norleucyl-l-norleucine, N-allyloxycarbonyl-, ethyl ester
24	24.952	3-Amino-1-azabicyclo[2.2.2]octane
25	26.264	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-
26	26.633	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-
27	26.700	Hexanedioic acid, bis(2-ethylhexyl) ester
28	27.909	Bis(2-Ethylhexyl) phthalate
29	28.573	1-benzylindole
30	29.369	1,4-Benzenedicarboxylic acid, bis(2-Ethylhexyl) ester

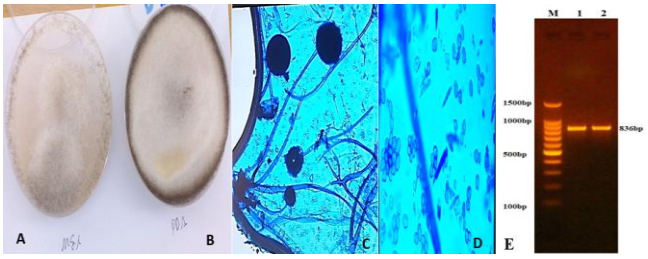

Figure 2: Morphological and molecular identification of *R. oryzae*. A: Growth on MEA. B: Growth on PDA. C: Sporangia and sporangiophores. D: Spores, E: Agarose gel electrophoresis image shows PCR product of fungal isolates.

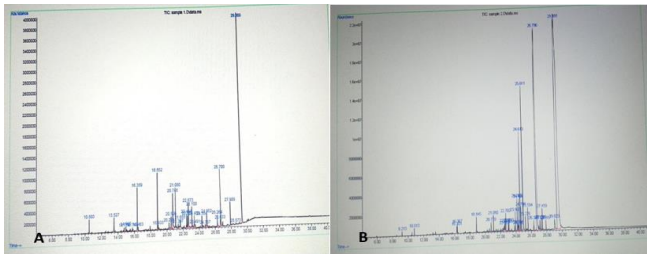

Figure 3: A: GC-MS analysis of crude extract produced from *R. oryzae* growing in CL medium, B: GC-MS analysis of crude extract produced from *R. oryzae* growing in CSL medium.

Table 3: GC-MA analysis of the compounds produced by *R. oryzae* using CSL medium

Peaks	RT	Compounds
1	9.2130	2-Ethyl-1-hexanol
2	10.813	1-Hexanol, 2-ethyl-
3	16.301	4-Tetradecene, (E)-
4	16.367	3-Tetradecene, (E)-
5	18.845	7-Hexadecene, (Z)-
6	20.770	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
7	21.080	1-Nonadecene
8	22.533	Pimelic acid, di(2-propyl) ester
9	22.570	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
10	22.702	Pimelic acid, di(2-propyl) ester
11	23.005	Adipic acid, isobutyl 2-octyl ester
12	23.093	5-Eicosene, (E)-
13	23.956	5-Octadecene, (E)-
14	24.207	Adipic acid, 2-decyl isobutyl ester
15	24.310	3,4-Furandicarboxylic acid
16	24.354	Methyl stearate
17	24.406	2-Ethylhexyl methyl isophthalate
18	24.613	Butyl citrate
19	24.701	1-Propene-1,2,3-tricarboxylic acid, tributyl ester
20	25.011	Butyl citrate
21	25.276	2-Butenedioic acid (E)-, bis(2-Ethylhexyl) ester
22	25.594	Butyl citrate
23	26.346	Hexanedioic acid, bis(2-Ethylhexyl) ester
24	26.796	Hexanedioic acid, bis(2-ethylhexyl) ester
25	27.128	Adipic acid, butyl 4-heptyl ester
26	27.290	Butyl citrate
27	27.459	Butyl citrate
28	27.917	Bis(2-Ethylhexyl) phthalate
29	29.023	1,3-Benzenedicarboxylic acid, bis(2-Ethylhexyl) ester
30	29.488	1,4-Benzenedicarboxylic acid, bis(2-Ethylhexyl) ester

Discussion

Rhizopus oryzae is a fungus with rapid growth and produces sporangiophores containing sporangia on their apexes. This morphological identification has been confirmed by molecular technique such as detecting MucL regions including their sequence and bioinformatics methods because no accurate technique in which the strains of *R. oryzae* can be classified depending on the producing organic acids (31-34). *R. oryzae* either pathogenic or saprophytic can produce different such as amylase, phytase, protase, lipase etc. and organic substances including acids Ghosh and Ray (8). This ability of the secreting enzymes can help utilize nutrient requirements including components of the

fermenting media leading to forming secondary metabolites at appropriate temperature degrees and incubating. For this reason, the present study may attribute the compounds produce from *R. oryzae* because the ability of the secreting enzymes against substrates and compositions of both CL and CSL media led to detect extracted compounds from that fungus using filtrated of the mentioned fermenting media.

Peeran *et al*., Researchers showed that the fungal secondary metabolites are different groups of compounds that participate in the fungus various biological functions such as competition, and symbiosis as well as these metabolites which can transport the metals (35). This idea of the heterogeneous metabolites and their functions can explain why the present study showed *R. oryzae* resulted in various compounds growing in two fermenting media containing metals, although similar compounds were characterized. Related to the enzymatic capability of this fungus, a strain of the *R. oryzae* synthesize tannin acylhydrolase which utilizes tannic acid to form gallic acid. This enzyme was produced when *R. oryzae* grew in the Czapex Dox medium supplemented with tannic acid, sodium nitrate, and glucose ratios, in which the tannase was also given (35). This result could support the results which used two synthetic media that *R. oryzae* was fermented and the produced compounds were detected by the same fungus and different media. Therefore, the capability of *R. oryzae* to form the enzymes may show that the extract of conocarpus leaves and corn powders participated as suitable substrates for giving the compounds. Additionally, magnesium, iron, and zinc have essential role in producing secondary metabolites of the fungi that the phosphates of the magnesium, iron, and zing participate in the formation of the co-enzymes, effects on cytochrome P450 oxidase, and stability of the protein components, respectively as well as utilization of the glucose and trace metals (15,36) which the used media of the current study applied. The additive substances were considered the remarkable growth factors of *R. oryzae* (37). Principally, the results of this study agreed with previous studies. Therefore, the medium is one of the most critical factors affecting secondary metabolites. *R. oryzae* is can assimilate various structural types of sugars such as glucose, galactose, mannose, pentose, arabinose, xylose, and hexose as well as metals, e.g., magnesium, phosphate, and calcium. Also, this fungus can utilize other trace metals. The constituents of a medium such as amino acids, metals, and vitamins have remarkable influence on microbial growth and the incubation period and temperature degree 25°C are also the significant by Frisvad (38). These results supported the present study producing compounds by *R. oryzae* fermented in two synthetic media. One of the most vital strategies for examining fungi infections is potassium chloride (KOH) mount, which is used as a foremost screening device (39). Some research suggests the pathological effects of the fungal ball in the bladder demonstrated the prognosis of aspergillosis. Moreover, it produces extreme poisonous secondary metabolites, which includes mycotoxin, mainly aflatoxins which labeled as carcinogenic by way of consumption of contaminated cereals like nuts and corns main to giant monetary losses (40). If no longer treated, these pathogenic microorganisms can also have a lethal impact on hosts and have a large have an impact on animal fitness, there are many recognized proteolytic enzymes generated from microorganisms (41).

Conclusions

This work concluded that the CL medium was suitable fermentation medium for *R. oryzae* in compared with CSL. However; furthermore studies can be used to test CL medium and its use for fermenting other fungal species.

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Conflict of interest

The authors declare no conflict of interest.

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الكشف عن المركبات المنتجة من فطر الرازبة الباسورزي

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الخلاصة

يصنف فطر الرازبة الباسورزي انه واحد من فطريات الميوكورالز وينتمي الى شعبة الزايكوماكوتا ويعتبر هذا الفطر مصدر لإنتاج عدد من المواد الطبيعية، مثلا كحول الايثانول، التمفا، حامض الفيومارك، حامض اللاكتيك وما يزال هذا الفطر يستخدم للحصول على الأنزيمات ومضادات الأحياء المجهرية فضلا عن الأحماض العضوية. في هذه الدراسة، تم استخدام أكار دكستروز البطاطس وأكار مستخلص الشعير لزراعة الفطر الرازبة الباسورزي تم التعرف على هذه الفطريات اعتمادا على المعلمات الشكلية واختبار تفاعل البلمرة التقليدي. تم استزراع الفطر في وسطين صناعيين هما سائل الذرة شديد الانحدار وأوراق كونوكاربوس عند ٢٥ درجة مئوية. أنتج الرازبة الباسورزي نموا غزيرا في كلا الوسطين وكان النمو أبيض رمادي. أظهر تحليل العديد من المركبات المختلفة في المستخلصات الخام من الرازبة الباسورزي باستخدام تلك الوسائط الاصطناعية المخمرة؛ ومع ذلك، تم توضيح عن بعض المركبات لتكون متشابهة في هياكلها. كانت المركبات المماثلة هي ميثيل بروبيل، ميثيل ستيرات، حمض هيكساندويك، ثنائي ٢-إيثيل هكسيل إستر، ثنائي ٢-إيثيل هكسيل فثالات، ١،٤-بنزيندیكاربوكسيليك أسيد. هدفت هذه الدراسة إلى تقييم وسط أوراق كونوكاربوس مقارنة مع سائل الذرة شديد الانحدار للكشف عن المركبات الطبيعية المنتجة من الرازبة الباسورزي.