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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2021.454.460

Research Article
Evaluation of Laccase Production by Monokaryotic Strains of Edible Mushrooms

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Abstract

Background and Objective: Edible mushroom laccases are one of the most attractive enzymes applicable in numerous industrial sectors. The purpose of this research is to construct monokaryotic strains from selected isolates of edible mushrooms and to study the effects of inducers on laccase production under solid-state fermentation. **Materials and Methods:** Isolation of local commercial strains of edible mushrooms was carried out from the pileus region using standard laboratory techniques. The laccase production was carried out using 40 mM 2,6-Dimethoxyphenol (2,6-DMP) and 40 mM guaiacol as substrate. The generation of monokaryotic strains was performed by mycelium homogenization in sterile water and regrowth in an appropriate medium. Laccase production and study of the effects of inducers on laccase production were then studied. **Results:** Laccase production of native and monokaryotic strains distinguished these strains into three groups: HIGH-(KK24, KK25), MEDIUM-(KK26, KK1, KK5 and KK23) and LOW (KK13, KK8). Reduced activity was found in almost all isolates after 14 days of inoculation. The effect of pure copper sulfate, copper sulfate with DMP, Tween80 and synthetic melanoidin was studied at 7 and 14 days. KK24 and KK25 showed their positive response to all inducers about 1.5-2.5 folds of activity to their native strains. **Conclusion:** Eight strains of local and commercial mushrooms were isolated and purified. The corresponding monokaryotic strains were generated from chemical dedikaryotization. Studies of laccase production showed that KK24 and KK25 were high laccase producer's throughout the incubation period. The addition of inducers augmented laccase activity in KK24 and KK25 along with their corresponding monokaryotic strains.

Key words: Mushroom, Laccase, edible mushroom, monokaryotic strains, solid-state fermentation, mycelium, guaiacum

Citation: Karittapattawan, P. and R. Benchawattananon, 2021. Evaluation of laccase production by monokaryotic strains of edible mushrooms. Pak. J. Biol. Sci., 24: 454-460.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Many edible mushrooms are prominent ligninolytic enzyme producers[1]. They fascinate not only mushroom producers, consumers but also researchers. Four remarkable species are *Agaricus bisporus* (white button mushroom), *Lentinula edodes* (Shiitake), *Pleurotus* spp. and *Volvariella volvacea*[2]. Mushroom production is widely promoted since it can accomplish a zero-waste plan and give benefits to enzyme productions. More than twenty mushroom species are cultivated in profit-making fashion in Thailand, among these, some rare species of *Pleurotus*[3].

Laccases are phenol oxidizing multicopper oxidases performing the oxidation associated with the four-electron reductions of molecular oxygen to water. A phenomenally broad substrate range adorned with low substrate specificity, laccases are attractive for numerous industrial and biotechnological sectors. Variabilities of laccase's application are as followed: used to remove phenol for product stability in beverage industries, paper mill preparation-deinking-effluent treatment in pulp industries and biodeterioration of the recalcitrant contaminated field in environmental industries[4,5]. Higher plants, most fungi, bacteria and insects produce laccases but fungi and mushrooms are the most studied laccase producers. Fungal laccases are well-known in ascomycetes and basidiomycetes[6].

The burden in mushroom or fungal laccase production is a result of a long fermentation period, low yield and unsatisfactory stability. Many attempts were carried out aiming to enhance laccase production employing genetic manipulations[7,8], co-cultivations, uses of inducers and optimization of growth conditions, etc[9,10]. Many types of research were performed focusing on the effects of artificial dedikaryotization in some basidiomycete's dikaryon. This technique was probably proposed as useful for enzyme production from robust neohaplonts.

The objectives of this research are to isolate local and commercial strains of edible mushrooms, to construct monokaryotic strains from selected isolates, to study the ability of laccase production in parent dikaryon and monokaryons and to study the effects of inducers on laccase production under solid-state fermentation.

MATERIALS AND METHODS

Study area: This research project was carried out in the Department of Microbiology and Department of Biological Science, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand from January, 2019-September, 2020.

Experimentation

Isolation of local-commercial strains of edible mushrooms: Some excised parts of the pileus region were washed several times in sterile distilled water and dried in a clean tissue towel before inoculation on Potato Dextrose Agars plates using standard laboratory techniques. The culture was incubated at 30±1°C for 6-12 days and observed for the mycelial growth at different intervals. Several sub-cultures were continued until pure cultures were obtained.

Construction of monokaryotic strains from selected isolates: To construct monokaryotic strains, dedikaryontization of mushroom was carried out following protocol published by Leal-Lara and Eger-Hummel[11]. An agar plug was cut from the edge of a 7-10 day old colony and transferred to a new sterile petri dish, 10 mL Potato Dextrose Yeast Broth was poured into the petri dish. On the 10th day after inoculation, a colony was divided into four parts using a sterile scalpel, three-quarters of the colony were homogenized at 22,000 rpm for 60 sec in 50 mL sterile distilled water. Total 10 μL aliquots were inoculated into 10 bottles of 50 mL sterile Peptone Glucose Solution, incubated at 28-30°C until mycelial conglomerates appeared from 24 hrs to 7 days. Any conglomerates found were homogenized again at 22,000 rpm for 60 sec in the same medium, 50 or 100 L aliquots were spread onto Potato Dextrose Yeast agar plates, incubated at 28-30°C until mycelial conglomerates appeared every 24 hrs. Every conglomerate was transferred to a new plate. Any colony showing no clamp connection was collected, named and characterized. Typification of monokaryons was realized by culturing fragments of monokaryons (0.5 cm plug) on the same Petri dish with Potato Dextrose Yeast Agar and incubated at 28-30°C. The presence of a clamp connection means that their nucleus was of a different type whereas the absence of a clamp connection means the monokaryons have a similar type of nucleus.

Assessment of laccase production: Mushrooms were cultured under solid-state fermentation using leaf litter as substrate. Laccase activity was assayed using 50 μL [40 mM DMP in 20 mM sodium acetate phosphate buffer pH 5.0] (ϵ_468 = 49,600 M^{-1} cm^{-1}, referred to DMP concentration), 350 μL distilled water and 100 μL crude enzyme extract. The concentration of the laccase enzyme was calculated by the formula:

$$C = \frac{A \times L}{\epsilon}$$

Where:

C = Concentration
A = Absorbance
L = Length of the cuvette (cm)

Inducers effect on laccase production by monokaryotic strains of edible mushrooms under solid-state fermentation: Selected monokaryotic strains of mushroom were cultivated under solid-state fermentation using leaf litter as a substrate with salt as found in common mushroom spawn used by mushroom growers. Copper sulfate was used as the main inducer in fixed concentrations. The effect of copper sulfate was studied in a combination of the presence of certain lignin monomers. Laccase activity was measured as described above.

All studies presented here are limited to some commercial mushroom strains found in the Khon Kaen province area, namely *Agrocybe aegerita*, *Agaricus bisporus*, *Hypsizygus marmoreus*, *Lentinus edodes*, *Pleurotus eryngii*, *P. ostreatus* and *P. sajors-caju*.

RESULTS

Isolation of local and commercial strains of edible mushrooms: Certain local and commercial strains of edible mushrooms were isolated. Processes of excision, washes and dry were effectuated on mushroom pileus regions. Mushroom sections were inoculated on Potato Dextrose agar plates at 30±1°C for 6-12 days. An apparition of mycelial growth was followed at different intervals. Several sub-cultures were made to obtain the pure culture of each type of mushroom. Eight strains were purified, collected and named as below (Table 1).

The assessment of laccase production was carried out by plate assay (Table 2). Laccase activity was measured at 7 days after inoculation by spectrophotometric methods using 40 mM 2,6-Dimethoxyphenol (2,6-DMP) and 40 mM guaiacol as substrate (Table 3).

Characterization of native, monokaryotic strains and efficiency of laccase production: A chemical dedikaryotization of mushrooms was carried out as followed. Briefly, an agar plug from the colony edge was subcultured in Potato Dextrose Yeast Broth for ten days (Fig. 1a). Three-quarters of the colony were homogenized and ten microliter aliquots were inoculated into sterile Peptone Glucose Solution. Mycelial conglomerates (Fig. 1b) were observed every 24 hrs to 7 days and were subjected to re-homogenized in sterile Peptone Glucose Solution and spread on Potato Dextrose Yeast agar plates. Mycelia were monitored every 24 hrs and transferred to a new container (Fig. 1c, d).

Laccase production assessment was effectuated both for native and selected monokaryotic strains at different time intervals under Solid-State Fermentation using leaf litter as substrate.

Assessment of laccase production: Laccase activity of native and selected monokaryotic strains was assayed at different time intervals using 2,6-Dimethoxyphenol (2,6-DMP) as a substrate (Table 4).

Table 1: Summary of pure mushroom isolates

Name	Species
KK26	*Agrocybe aegerita*
KK1	*Agaricus bisporus*
KK5	*Hypsizygus marmoreus*
KK23	*Hypsizygus marmoreus*
KK8	*Lentinula edodes*
KK25	*Lentinus squarrosulus*
KK13	*Pleurotus eryngii*
KK24	*Pleurotus ostreatus*

Table 2: Presumptive test of laccase production on solid media supplemented with 2,6-Dimethoxyphenol (2,6-DMP) and guaiacol

	Diameter of coloring zone (cm) during the time course (days)							
	2,6-DMP				Guaiacol			
Names	Day 1	Day 3	Day 5	Day 7	Day 1	Day 3	Day 5	Day 7
KK26	1.03	1.70	1.73	1.80	1.60	1.80	2.03	2.20
KK1	2.00	2.83	3.53	9.00	1.80	2.23	2.60	3.20
KK5	2.30	2.73	3.40	9.00	2.20	2.60	2.90	3.40
KK23	2.00	2.30	2.90	3.40	2.00	2.13	2.13	2.15
KK8	2.00	2.43	3.10	3.53	1.70	1.83	2.23	2.60
KK25	2.60	2.73	3.80	3.83	2.60	2.60	2.60	2.60
KK13	0.60	0.80	0.80	0.83	0.70	0.73	0.80	0.90
KK24	1.10	1.13	1.43	1.70	1.30	1.30	1.40	1.40

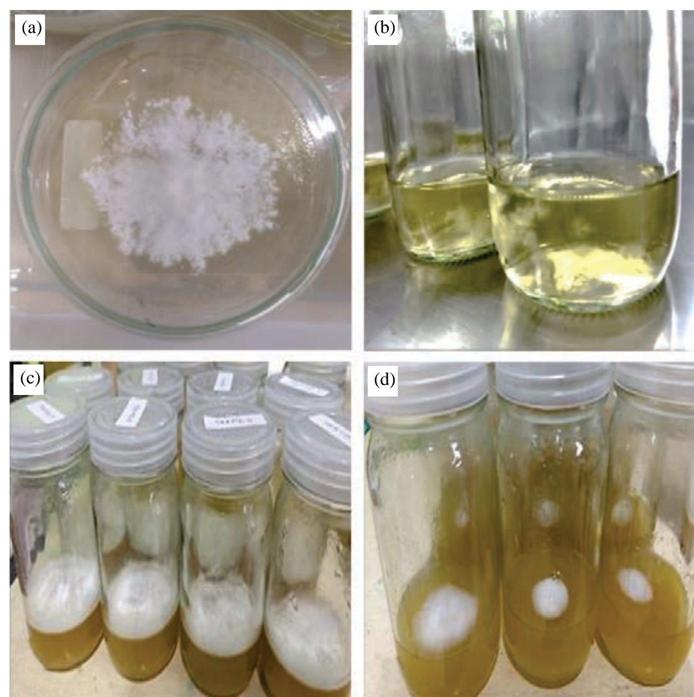

Fig. 1(a-d): Certain steps from dedikaryotization process
A: Mushroom colony at day 10 after inoculation in Potato Dextrose Yeast Broth, B: Mycelial conglomerates reappeared after incubation of aliquot of the homogenized colony in sterile Peptone Glucose Solution, C and D: Monokaryotic strain of KK24 and KK25

Table 3: Spectrophotometric assay of laccase production in liquid media supplemented with 2,6-Dimethoxyphenol (2,6-DMP) and guaiacol

	Laccase activity (U mL^{-1})	
Names	2,6-DMP	Guaiacol
KK26	93.75	87.15
KK1	81.15	73.99
KK5	101.51	119.66
KK23	108.57	93.27
KK8	74.09	75.20
KK25	433.27	411.66
KK13	77.32	76.61
KK24	432.76	418.55

Table 4: Laccase activity of native and selected neohaplonts strains at different times

	Laccase activity (U mL^{-1}) during the time course (days)									
Names	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 21	Day 28
KK26	67.34	67.34	70.67	75.20	94.76	105.75	113.61	112.80	81.05	247.18
KK1	67.34	67.44	71.98	76.61	81.15	93.15	119.76	94.25	89.72	108.17
KK5	73.69	70.97	92.54	101.51	130.14	129.54	155.75	229.23	148.89	144.25
KK23	78.63	80.14	92.14	108.57	119.66	135.58	197.68	169.46	144.15	118.85
KK8	66.33	65.12	73.99	74.09	69.25	73.99	78.83	76.11	76.71	78.33
KK25	70.67	198.08	360.69	569.96	543.35	186.49	209.27	186.39	223.99	175.30
KK13	72.48	73.79	74.60	75.20	75.00	77.32	79.94	81.35	79.94	84.27
KK24	69.86	96.57	312.90	432.76	418.55	241.53	221.37	201.01	220.77	247.18
mKK26	68.75	80.65	95.06	94.46	97.68	167.54	187.60	266.33	116.53	243.75
mKK1	70.67	74.80	71.57	123.49	87.70	113.21	128.63	246.57	122.28	243.85
mKK5	70.77	81.35	79.13	87.30	92.64	98.69	125.81	144.15	178.23	181.35
mKK23	79.94	77.52	79.94	80.14	81.96	85.99	84.27	88.51	96.17	107.36
mKK8	68.75	67.64	68.65	70.06	68.75	71.77	75.91	78.93	75.10	76.21
mKK25	70.46	81.05	174.50	81.96	118.65	82.36	77.42	105.54	127.62	98.79
mKK13	71.67	76.92	77.52	79.94	82.26	84.98	83.37	84.27	96.77	87.70
mKK24	69.56	76.61	74.70	74.50	75.20	68.85	73.29	69.66	71.57	75.60

Table 5: Laccase activity in presence of laccase inducers at 7 and 14 days after inoculation

	W/O		Copper		Copper+DMP		Copper+Tween80		Copper+syn M.	
	Days		Days		Days		Days		Days	
Names	7	14	7	14	7	14	7	14	7	14
KK26	86.98	115.21	106.99	109.44	147.87	172.81	139.17	173.96	83.50	118.66
KK1	83.38	102.01	118.40	156.07	133.41	142.81	141.75	183.61	83.38	99.96
KK5	110.83	172.49	145.18	213.89	144.07	206.99	130.77	189.74	109.72	170.77
KK23	112.62	183.57	129.51	216.61	111.49	220.28	112.62	223.96	109.24	183.57
KK8	74.17	77.47	80.10	85.22	114.22	131.70	118.67	108.46	72.69	77.47
KK25	506.66	193.33	780.25	309.33	1129.84	483.33	1215.97	444.66	658.65	251.33
KK13	77.10	80.65	87.89	96.77	84.81	104.84	100.23	104.84	77.10	80.65
KK24	400.66	206.19	556.91	329.90	1081.77	536.09	885.45	536.09	641.05	309.29
mKK26	96.07	206.97	113.36	231.80	144.11	289.75	153.71	289.75	94.15	206.97
mKK1	95.60	157.60	126.19	187.54	152.95	267.92	145.30	220.64	98.46	152.87
mKK5	87.97	134.98	111.72	175.47	131.96	187.62	123.16	161.98	91.49	137.68
mKK23	81.05	86.39	87.53	89.85	94.83	120.95	97.26	95.03	72.95	77.75
mKK8	69.41	77.42	77.73	85.16	97.17	108.39	90.23	92.90	72.18	69.68
mKK25	95.31	96.48	165.83	154.37	219.20	250.85	181.08	173.66	142.96	144.72
mKK13	81.10	83.82	88.40	92.20	97.32	108.97	105.43	117.35	82.72	83.82
mKK24	74.85	72.48	145.21	144.23	194.61	188.44	149.70	130.46	119.76	108.71

W/O: Without, syn M.: Synthetic melanoidin, DMP: 4,6-dimethyl-2-mercaptopyrimidine

Table 6: Fold of laccase activity in presence of laccase inducers

	W/O		Copper		Copper+DMP		Copper+Tween		Copper+syn M.	
	Days		Days		Days		Days		Days	
Names	7	14	7	14	7	14	7	14	7	14
KK26	1	1	1.2	1	1.7	1.5	1.6	1.5	1	1
KK1	1	1	1.4	1.5	1.6	1.4	1.7	1.8	1	1
KK5	1	1	1.3	1.2	1.3	1.2	1.2	1.1	1	1
KK23	1	1	1.1	1.2	1.0	1.2	1.0	1.2	1	1
KK8	1	1	1.1	1.1	1.5	1.7	1.6	1.4	1	1
KK25	1	1	1.5	1.6	2.2	2.5	2.4	2.3	1.3	1.3
KK13	1	1	1.1	1.2	1.1	1.3	1.3	1.3	1	1
KK24	1	1	1.7	1.6	2.7	2.6	2.2	2.6	1.6	1.5
mKK26	1	1	1.2	1.1	1.5	1.4	1.6	1.4	1	1
mKK1	1	1	1.3	1.2	1.6	1.7	1.5	1.4	1	1
mKK5	1	1	1.3	1.3	1.5	1.4	1.4	1.2	1	1
mKK23	1	1	1.1	1.1	1.2	1.4	1.2	1.1	0.9	0.9
mKK8	1	1	1.1	1.1	1.4	1.4	1.3	1.2	1.0	0.9
mKK25	1	1	1.7	1.6	2.3	2.6	1.9	1.8	1.5	1.5
mKK13	1	1	1.1	1.1	1.2	1.3	1.3	1.4	1	1
mKK24	1	1	1.9	2.0	2.6	2.6	2.0	1.8	2.0	1.8

W/O: Without, syn M.: Synthetic melanoidin, DMP: 4,6-dimethyl-2-mercaptopyrimidine

Inducers effect on laccase production by monokaryotic strains of edible mushrooms under solid-state fermentation: The effect of pure copper sulfate, copper sulfate with DMP, Tween80 and synthetic melanoidin was studied at 7 and 14 days (Table 5). A fold of change in laccase activity was calculated at day 7 and 14 (Table 6).

DISCUSSION

Eight commercial strains of edible mushrooms were isolated, named and subjected to test their ability to produce laccase both by plate assay and spectrophotometric method assay. KK25 and KK24 were exceptional laccase producers among isolates. All isolates were undergone monokaryotization by chemical dedikaryotization and opted for corresponding strains of each. This method was used to create a series of monokaryotic strains of model organisms like many species of *Pleurotus* and *Lentinula*[12,13]. Laccase activity of native and selected monokaryotic isolates was studied at different time intervals under solid-state fermentation supplemented with 2,6-Dimethoxyphenol (2,6-DMP). Many monokaryotic isolates showed laccase activity in a quasi-

identical way to their parent strains except for those derived from KK25 and KK24. Other cases could encounter less, equal or more activity in monokaryotic derivative strains[14,15]. The addition of supplements such as copper ions or lignin monomer had known effects of the increasing quantity of secreted enzymes[16,17]. Here, the effect of copper sulfate, copper sulfate with DMP, Tween80 and synthetic melanoidin was studied at 7 and 14 days. The fold of increase in laccase activity was monitored between 1.1-2.7 fold compared to non-induced condition. Although, it was reported a higher fold of increase in laccase activity, 8.6 fold, in the case *Lentinus tigrinus*. This could be due to the diversity and variability of laccases produced from white-rot fungi[16]. While many types of research focused on various lignocellulosic waste such as citrus peels, banana peels, peach waste etc[18], mixed leaf litter was used in this study. Although it has less lignocellulosic extent for basal laccase production, it could eventually be developed as an economic substrate for laccase production under solid-state fermentation[19, 20]. DMP and Tween80 seem to accentuate the copper inducer. Yet it has been shown that non-ionic surfactant could promote the secretion of the enzyme[18,21]. Melanoidins, complex polymers formed from the heating process of reducing sugars and proteins called Maillard Reaction (MR) are commonly found in sugar cane molasses from fermentation in sugar industries. Melanoidins lead too many environmental problems like increased BOD, inhibition of vegetation especially seed germination. Many approaches had been tried for melanoidin removal from waste water[22]. It has been shown its laccase inducing capacity[23]. The diminishing effect of synthetic melanoidin could be both by its complex structure generated from subsequent reactions during the stage of non-enzymatic formation and reason of its antioxidant property[24]. The study of laccase induction by synthetic melanoidin could ameliorate its risks to the environment.

CONCLUSION

Eight strains of local and commercial mushrooms were isolated and purified. Monokaryotic strains can be developed by chemical dedikaryotization. The corresponding monokaryotic strains were generated from chemical dedikaryotization. Monokaryotic strains of tested mushrooms did behave in the quasi-identical to their dikaryotic parents. Studies of laccase production showed that KK24 and KK25 were high laccase producers throughout the incubation period. The addition of inducers augmented laccase activity between 1.5-2.5 fold in KK24 and KK25 along with their corresponding monokaryotic strains.

SIGNIFICANCE STATEMENT

This study discovers that the monokaryotic mushroom strain generation was feasible. That can be a benefit for laccase production from mushroom, culture maintenance with less genetic impairment. This study will help the researcher to uncover the critical areas of laccase production that many researchers were not able to explore. Thus a new theory on the inducer combination may be arrived at.

ACKNOWLEDGMENT

The authors would like to offer particular thanks to the Biological Science Faculty of Science Khon Kaen University for laboratory facilities.

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