Improvement of Cellulolytic Activity of *Pleurotus florida* through Radiation Mutagenesis

Chandran Sathesh-Prabu and Young-Keun Lee*

Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Korea

Abstract - A mushroom mutant with increased cellulolytic activity was developed through radiation mutagenesis. The homogenized hypha suspension of *Pleurotus florida* was exposed to gamma radiation (^{60}Co, AECL) at the dose of LD_{99} (0.51 kGy, D_{10}; 0.26 kGy). Among 16 mutants, Pf CM4 showed 17.24% more cellulolytic activity than the wild type ($p<0.05$). It was observed that Pf CM4 can utilize all kinds of carbon sources tested for their mycelia growth. Starch, xylan, and glucose favourably supported the radial mycelia extension. Yeast extract and NH_4NO_3 have been recorded as the best organic and inorganic nitrogen sources, respectively. Pf CM4 was found to grow significantly faster, even at high temperature (30°C), than wild type ($p<0.05$), and the optimal pH was 5.5～6.5. This study reveals that the mutant Pf CM4 could be employed for the effective recycling of cellulosic wastes, in addition to mushroom farming.

Key words : Cellulase, Mushroom, *Pleurotus florida*, Radiation mutagenesis

INTRODUCTION

Mushrooms, the fruiting bodies of macrofungi, are simply known as a protein rich vegetable, and have been used for their gastronomic and medicinal values by mankind for thousands of years all around the world. Mushrooms can also degrade virtually any lignocellulosic waste and play a vital role in disposing of a lot of waste including domestic, industrial, and agricultural waste (Okhuoya 2011). Brodkorb and Legge (1992), Heinfling *et al.* (1997) and Knapp *et al.* (1997) observed that mushrooms are more efficient than bacteria in the biodegradation of a number of recalcitrant compounds such as xenobiotics owing to their extracellular enzyme system. Oyster mushrooms (*Pleurotus* spp.) occupy the second place in the world's production of edible mushrooms, after the popular white button mushroom, *Agaricus bisporus* (Kues and Liu 2000; Berne *et al.* 2008), and the genus *Pleurotus* comprises about 40 species (Jose and Janardhanan 2000). Lignocellulose, the principal component of the plant cell wall, accounts for about 50% of the biomass in the world with an estimated annual production of $10 \sim 50 \times 10^{12}$ kg (Classen *et al.* 1999), and it is mostly wasted in the form of post-harvest agricultural residue and the waste from food processing industries (Mtui 2009). This natural, potentially cheap, and abundant polymer can be effectively utilized for the cultivation of mushrooms as the bioconversion of agriculture and industrial waste into food has attracted the world's attention in recent years (Ingale and Ramteke 2010). Mushroom cultivation is a highly efficient method of disposing agro-lignocellulosic waste as well as producing nutritious food (Cohen *et al.* 2002), and this in turn can minimize the environmental pollution owing to unutilized agricultural waste as well as reduce air pollution associated with the burning of agricultural waste (Ahmed *et al.* 2009). As cellulose is one of the major components of lignocelluloses, efforts should be made to obtain high levels of cellulose degrading

* Corresponding author: Young-Keun Lee, Tel. +82-63-570-3300, Fax. +82-63-570-3309, E-mail. yklee@kaeri.re.kr

enzymes producing mushroom strains to potentially utilize cellulose waste. Mutation breeding is a process by which mutant variants with desirable traits to be bred are developed, without altering the remaining genotype, by physical, chemical and biological mutagenic agents (Sathesh-Prabu and Lee 2011). Gamma-ray radiation can change the genetic diversity of filamentous fungi and induce positive mutants (Boominathan *et al.* 1990). Among 242 mutant varieties, about 61% of the mutants were induced by gamma-ray irradiation in Japan indicating that mutation breeding through gamma-ray irradiation is an effective and highly successful approach for the generation of commercial cultivars (Nakagawa 2009). In this lime light, the present investigation was undertaken to develop *Pleurotus* spp. mutants with enhanced cellulose degrading activity through gamma radiation induced mutagenesis.

MATERIALS AND METHODS

1. Chemicals

All chemicals and microbiological media were purchased from Sigma-Aldrich and Difco, respectively. All chemicals used were of analytical grade unless otherwise stated.

2. Strains

The pure cultures of *Pleurotus* spp. such as *P. eryngii* (KACC 42719), *P. sajor caju* (KACC 42720), *P. ostreatus* (KACC 42738), and *P. florida* (KACC 52110) were procured from the Korean Agricultural Culture Collection Centre, Suwon, Korea. The cultures were maintained in a potato dextrose agar (PDA) medium at 4˚C and sub-cultured at 30-day intervals.

3. Radiation sensitivity of mushroom strains

Mycelia of four mushroom strains were collected by centrifuging 15 ml of seven-day old cultures grown in potato dextrose broth and washed twice with sterile distilled water. An aliquot (1.5 ml) of hypha suspension with sterile distilled water, prepared through the homogenization of mycelia by a homogenizer (Polytron PT 1200E, Kinematica AG), was used for radiation sensitivity studies. The homogenized hypha suspension was irradiated in a ^{60}Co irradiator (dose rate 920 Gy h^{-1}, AECL) at 0.05 to 1 kGy at the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Korea. The dose rate was determined through alanine dosimetry (EPR (e-scan), Bruker). The hypha survival rate in the irradiated samples was determined using serial dilution and a plate count method. The D_{10}-value was determined by plotting the radiation dose (kGy) on the X-axis, and the survival ratio on the Y-axis. The negative reciprocal of the slope indicated the D_{10}-value. The LD_{99}-value was determined using the formula, LD_{99}=log(0.01). $slope^{-1}$.

4. Mutagenesis and screening of cellulase mutants

To induce the radiation mutagenesis, the hypha suspension was prepared as described above, and exposed to gamma radiation at the dose of LD_{99}. Irradiated samples were plated onto a cellulase mutant screening medium (CMSM). CMSM contained Buswell mineral salt medium (BMSM, Buswell *et al.* (1996)) and 0.1% ($v.v^{-1}$) triton X 100 and 4 $g.l^{-1}$ sorbose to enable the screening of a large number mutants (Chand *et al.* 2004). BMSM contained ($g.l^{-1}$): KH_2PO_4, 1.0; K_2HPO_4, 0.4; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.013; yeast extract, 0.1; L-asparagine, 1.5; NH_4NO_3, 0.5; thiamine.HCl, 0.0025; agar, 15; and 1 ml of a trace element solution consisting of ($g.l^{-1}$): ferric citrate, 4.8; $ZnSO_4.7H_2O$, 2.64; $MnCl_2.4H_2O$, 2.0; $CoCl_2.6H_2O$, 0.4 and $CuSO_4.5H_2O$, 0.4. Carboxymethylcellulose (CMC) (1%) served as the carbon source. Six different CMSMs (CMSM I to CMSM VI) were prepared to obtain the potent mutants and to understand the growth pattern of mushroom strains in irradiated samples. The composition of CMSM I to CMSM VI were as follows: CMSM I (1% CMC+0.4% Sorbose+0.1% Triton X 100−Glucose); CMSM II (1% CMC−Sorbose−Triton X 100−Glucose); CMSM III (1% CMC+Sorbose+Triton X 100+0.5% Glucose); CMSM IV (1% CMC−Sorbose−Triton X 100+0.5% Glucose); CMSM V (1% CMC+0.4% Sorbose+0.1% Triton X 100+1% Glucose); and CMSM VI (1% CMC+0.4% Sorbose+0.1% Triton X 100+2% Glucose). The plates were incubated at 25˚C for 7～10 days. All the mutants formed only on CMSM I were taken for further studies, and pure cultures were made.

5. Cellulolytic activity of mutants

The cellulolytic activity of the selected mutants was studied

on the above mentioned BMSM with 1% CMC as the sole carbon source. Mycelium agar plugs (six mm in radius) of the respective mutants were punched out from the margin of an actively growing colony (seven days old) using a sterile cork borer and inoculated in the center of the CMC amended BMSM medium. The plates were incubated at 25°C for 144 h. Mycelia growth (radial extension, *Rme*) was measured in eight radius lengths of the colony from the centre of the inoculum to the periphery of the colony at 24 h intervals for 144 h. Various growth kinetics of mutants such as colony radial growth (*Kr*), specific growth rate (α), peripheral growth zone (*w*), and percent of growth deviation were calculated as described elsewhere (Trinci 1971; Baumer 2008). Those mutants that showed better degradation activity over the wild type were selected for further studies.

6. Growth of mutant on different carbon and nitrogen sources

To study the utilization of different carbon and nitrogen sources by the mutant, the BMSM was prepared separately with 1% ($w.v^{-1}$) of each cellulose, xylan, starch, pectin, chitin, sorbose, and glucose, and 0.2% ($w.v^{-1}$) of each yeast extract, beef extract, tryptone, peptone, casaminoacids, L-asparagine, NH_4Cl, NH_4NO_3, $NaNO_3$, $(NH_4)_2SO_4$ and urea, respectively. The growth of the selected mutant on a PDA was also evaluated. The selected mutant was inoculated in the respective medium and incubated at 25°C for 144 h. Mycelia growth was measured, as described above, after 144 h of incubation. Mycelia density was also observed visually and categorized into five groups: very scanty (1+), scanty (2+), moderate (3+), dense (4+), and very dense (5+).

7. Effect of different temperatures and initial medium pH on mutant growth

The PDA medium was prepared ($39\ g.l^{-1}$) and the pH of the medium was adjusted to be within the range of 5.0 to 9.0 at 0.5 pH intervals with 1 M HCl and 1 M NaOH using a bench top pH meter (520A, Orion). After inoculation, the respective plates were incubated at 25°C for 144 h. To study the suitable temperature for the mycelia growth of the mutant, the selected mutant was inoculated in the PDA medium, and the plates were incubated at different temperatures (18, 20, 22, 24, 25, 26, 28, 30 and 32°C) for 144 h. Mycelia growth was measured, as described earlier, after 144 h of incubation.

8. Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) followed by a Tukey test and a t-test using an SPSS-20 package to determine the level of significance of the variants in all experiments caused by the variables studied. The probability values (p) of < 0.05 or < 0.01 were considered significant.

RESULTS AND DISCUSSION

The radiation sensitivity of mushroom strains was studied to determine the D_{10} and LD_{99} values. The D_{10}-value refers to the radiation dose required to effect a 90% reduction in colony forming units. *P. ostreatus* recorded the highest D_{10} value (0.45 kGy), followed by *P. sajor caju* (0.36 kGy), *P. eryngii* (0.28 kGy), and *P. florida* (0.26 kGy). The same trend was also observed in case of LD_{99}-values (Table 1). Among the four strains, *P. florida* was found to be the most radiation sensitive strain. The reason for the low D_{10} and LD_{99} values may be due to the use of mycelia (Mechanical homogenizer may cause cell wall breakage) for the radiation studies, which was found to be more radiation sensitive than basidiospores (Lee and Chang 1999).

A homogenized hypha suspension was irradiated at the dose of LD_{99} to obtain the mutants. The mutants formed in the CMSM I were considered as potent mutants because CMSM I was devoid of glucose, but supplemented with growth restrictors such as sorbose and triton X 100. Sorbose is an inhibitor of 1,3-β glucan biosynthesis (Bisaria *et al.* 1986). Triton X 100 was used to limit the colony size, thus enabling the screening and isolation of mutants (Gadgil 1995; Jiang *et al.* 2011). *P. ostreatus* produced 5, 36, 6, 48, 7, and 7 mutants in CMSM I to CMSM II, respectively. Though *P. eryngii* gave only one potent mutant in CMSM I, but no mutants were observed in CMSM III, V and VI. *P. eryngii*

Table 1. Radiation sensitivity of *Pleurotus* spp.

Strains	Radiation sensitivity (kGy)		
	LD_{99}	Equation of the linear line	D_{10}
Pleurotus florida	0.51	$Y=-3.884x+0.053$	0.26
Pleurotus eryngii	0.56	$Y=-3.553x+0.108$	0.28
Pleurotus sajor caju	0.72	$Y=-2.789x+0.289$	0.36
Pleurotus ostreatus	0.89	$Y=-2.241x-0.124$	0.45

gave the highest number of mutants (108) in CMSM IV compared with *P. ostreatus* (48), *P. sajor caju* (62), and *P. florida* (34). *P. florida* gave 6, 16, 7, 34, 6, and 7 mutants in CMSM I to CMSM VI, respectively. The number of mutants formed by *P. sajor caju* were 4, 40, 3, 62, 5, and 6 in CMSM I to CMSM VI, respectively. The results of mutant screening in six different CMSMs showed that the addition of glucose did not show any significant effect on the growth or formation of mutants ($p < 0.05$) except an increase in the mycelia density. On the other hand, the addition of sorbose and triton X 100 influenced the growth of mutants, which in turn facilitated the isolation of the potent mutants in the irradiated samples ($p < 0.05$). In the present study, it was observed that even though *P. florida* was found to be the most radiation sensitive strain among the four mushroom strains taken for the study, but it gave maximum number of potent mutants in CMSM I compared with the other strains. A total of 16 potent mutants were obtained from four strains of mushrooms (*P. florida* (6), *P. ostreatus* (5), *P. sajor caju* (4), and *P. eryngii* (1)) and all of them were taken for a cellulolytic activity assay.

The cellulolytic activity of the selected mutants was evaluated in BMSM supplemented with 1% ($w.v^{-1}$) CMC as the sole carbon source. Table 2 describes the various growth kinetic parameters such as radial mycelia extension (*Rme*), colony radial growth (*Kr*), specific growth rate (α), peripheral growth zone (*w*), and percent of growth deviation of the selected mutants and their respective wild types. It was found that of 16 potent mutants; only one mutant (Pf CM4) recorded a positive growth percentage (17.24%) showing the maximum cellulolytic activity over the wild type ($p < 0.05$). All the growth kinetic parameters obtained with Pf CM4 were higher than that of the other mutants. It is clear that Pf CM4 was the most efficient mutant in degrading cellulose in terms of CMC. Therefore, Pf CM4 was selected for further studies. This characteristic (increased cellulolysis) was found to be stable at up to four generations of sub-culturing. Lee *et al.* (2000) developed lignocellulolytic mutants of *P. ostreatus* by irradiating the mycelia with gamma-rays (1 ~ 2 kGy) and found that the extracellular enzymes of the mutants were up

Table 2. Growth kinetics of *Pleurotus* spp. and their respective mutants on CMC medium

Mutants	Radial mycelia extension (*Rme*, mm)[1]	Colony radial growth (*Kr*, $\mu m.h^{-1}$)[2] (Linear Model)	Specific growth rate (α, h^{-1})[3] (Exponential Model)	Peripheral growth zone (w, $\times 10^3 \mu m$)[4]	Percent growth increase or decrease (over control)
Pe[5]	24.56 ± 0.09	119.00 ± 14.10	0.009 ± 0.001	14.04 ± 1.00	0
Pe CM1	12.75 ± 0.88**	49.00 ± 8.48**	0.006 ± 0.001	8.88 ± 0.40**	−48.09
Ps[6]	28.81 ± 0.97	164.00 ± 4.24	0.011 ± 0	14.91 ± 0.39	0
Ps CM1	[a]17.19 ± 0.09*	79.00 ± 1.41**	0.007 ± 0	11.29 ± 0.20*	−40.35
Ps CM2	[a]18.38 ± 0.53*	88.50 ± 3.54**	0.008 ± 0	11.06 ± 0.44*	−36.23
Ps CM3	[a]18.56 ± 0.80*	90.50 ± 6.36**	0.008 ± 0	11.31 ± 0.80*	−35.57
Ps CM4	[a]18.56 ± 0.44*	87.50 ± 4.95**	0.008 ± 0.71	11.69 ± 0.44*	−35.57
Po[7]	32.56 ± 0.80	183.50 ± 7.78	0.012 ± 0.01	15.97 ± 0.31	0
Po CM1	[a]17.31 ± 1.33*	75.00 ± 8.49**	0.007 ± 0	10.71 ± 1.21*	−46.83
Po CM2	[a]17.63 ± 1.41*	77.50 ± 6.36**	0.007 ± 0	11.07 ± 0.91*	−45.87
Po CM3	[a]19.56 ± 1.68*	89.30 ± 11.31**	0.008 ± 0.71	11.85 ± 0.39	−39.92
Po CM4	[a]21.69 ± 4.34	117.50 ± 36.06*	0.009 ± 1.41	12.90 ± 1.98	−33.40
Po CM5	[a]29.25 ± 3.89	154.50 ± 27.58*	0.010 ± 1.41	15.41 ± 0.58	−10.17
Pf[8]	29.00 ± 3.01	158.00 ± 19.80	0.011 ± 0.001	14.37 ± 0.05	0
Pf CM1	[a]21.69 ± 1.68	110.00 ± 12.73	0.009 ± 0	12.22 ± 1.41	−25.22
Pf CM2	[a]27.13 ± 1.41	148.00 ± 7.78	0.011 ± 0	13.50 ± 0.71	−6.47
Pf CM3	[a]19.50 ± 5.30*	95.50 ± 36.06*	0.009 ± 0.002	11.05 ± 1.48*	−32.76
Pf CM4	[b]34.00 ± 0.53*	201.50 ± 2.12*	0.013 ± 0	15.50 ± 0.16*	17.24
Pf CM5	[a]25.81 ± 1.33	139.00 ± 7.07	0.011 ± 0.001	13.25 ± 0.22	−10.99
Pf CM6	[a]19.25 ± 2.47*	93.50 ± 20.51*	0.008 ± 0.001	11.64 ± 0.51*	−33.62

[1]measured in eight radius lengths of a colony from the centre of the inoculums after 144 h of inoculation.

[2]slope of the model or $Kr = (r_1 - r_0)/(t_1 - t_0)$. r_1 = colony radius at time t_1. r_0 = colony radius at time t_0

[3]$r = r_o\, e^{\alpha t}$. α = specific growth rate. r = radius in mm. t = time in hours.

[4]w = peripheral growth zone (region in which hyphae are able to contribute protoplasm to the apical extension of the colony's leading hyphae). $w = Kr/\alpha$

[5]*P. eryngii*, [6]*P. sajor caju*, [7]*P. ostreatus*, [8]*P. florida* ([5] to [8] all wild type)

Values are expressed as means ± SD of triplicates. Same superscript letter refers no significant difference among mutants within group.

* and ** denote the significant difference over respective wild type at the level of p = 0.05 and 0.01 respectively.

to 10-times higher than the control. From these results, it seems that the gamma-ray radiation induces the mutants that could be used to recycle the lignocellulosic wastes.

The utilizations of different carbon and organic and inorganic nitrogen sources by Pf CM4 were studied. The radial mycelia extension (*Rme*), colony radial growth (*Kr*), and mycelia density of Pf CM4 and the wild type for various carbon sources are given in Table 3. It was observed that apart from CMC, Pf CM4 can utilize all kinds of carbon sources including polysaccharides in addition to sorbose, an inhibitor of cell wall synthesis. It was found that radial mycelia extension was significantly supported by starch, xylan, and glucose, and the opposite was recorded in sorbose and chitin as a carbon source. Sorbose almost completely inhibited the growth of the wild type. All sugars were effectively utilized by Pf CM4 compared with the wild type ($p < 0.05$). In the present study, it was found that Pf CM4 preferred polysaccharides (xylan and starch) more than glucose ($p > 0.05$). This result was consistent with the results of Jaindaik and Kapoor (1976), who reported that starch supports the growth of oyster mushrooms. This result is contrary, but partially similar to Anyakorah *et al.* (2004), who reported that *P. ostreatus* prefers polysaccharide, cellulose, rather than monosaccharides such as glucose and mannose. The results of the present study indicate that Pf CM4 tends to grow on a wide range of carbon sources, even on complex sugars. The radial mycelia extension (mm) of Pf CM4 and the wild type on PDA were 13.25 ± 0.35 and 17.20 ± 2.71. *i.e.*, 29.81% more mycelia growth occurred in Pf CM4 than in the wild type. Djayanegara and Harsoyo (2009) obtained radiation-induced mutants of *P. florida*, which showed faster mycelia growth compared to the control.

In the case of nitrogen sources, Pf CM4 preferred organic nitrogen more than inorganic nitrogen (Table 4). This result is consistent with the results of earlier studies on various *Pleurotus* spp. (Song *et al.* 2001; Qinnghe *et al.* 2004; Imtiaj *et al.* 2009). Among organic nitrogen, Pf CM4 showed the maximum growth and mycelium density in yeast and beef extracts. Even though L-asparagine gave similar values of colony radial growth rate as for yeast and beef extracts, it was observed that the mycelia density was only half of that of both extracts. Pf CM4 followed a yeast extract ≥ beef extract > tryptone > peptone > casaminoacids > L-asparagine sequence for the mycelia growth. It was observed that inorganic nitrogen also favoured the growth of Pf CM4 as organic nitrogen, but the mycelia density was not as high as organic nitrogen. This could be explained by the limited nutrition in the growth media. In the case of inorganic nitrogen, Pf CM4 followed a $NH_4NO_3 > (NH_4)_2SO_4 > NH_4Cl >$ Urea $> NaNO_3$ sequence. $NaNO_3$ could be little utilized by Pf CM4 as a nitrogen source. In contrast, the wild type preferred the urea rather than NH_4NO_3, and followed a urea $> NH_4Cl > NaNO_3 \geq (NH_4)_2SO_4 > NH_4NO_3$ sequence. This observation is in accordance with the results of Adebayo-Tayo *et al.* (2011) who observed that the maximum mycelia growth of *P. ostreatus* was achieved when urea was used as the sole nitrogen source. The most probable reason for the preferential utilization of organic nitrogen over inorganic nitrogen is the accessibility of amino acids in organic nitrogen, as organic nitrogen contains all kinds of amino acids, which can be absorbed directly by the mycelia (Qinnghe *et al.* 2004). Hwang *et al.* (2003) stated that certain essential amino acids could not be synthesized from inorganic nitrogen sources.

In the present study, it was observed that the mycelia

Table 3. Growth of Pf CM4 on various carbon sources

Carbon sources	Wild type			Pf CM4		
	Radial mycelia extension (*Rme*, mm)	Colony radial growth rate (*Kr*, μm.h^{-1})	Mycelia density	Radial mycelia extension (*Rme*, mm)	Colony radial growth rate (*Kr*, μm.h^{-1})	Mycelia density
Cellulose	14.63 ± 1.06^{a}	58.16 ± 12.50	1+	24.25 ± 4.6^{a}	126.74 ± 31.92	2+ ~ 3+
Xylan	26.50 ± 1.77^{b}	142.36 ± 12.28	2+	32.88 ± 4.77^{a}	186.63 ± 33.15	3+
Starch	27.19 ± 3.80^{b}	147.14 ± 26.39	3+	33.88 ± 3.77^{a}	193.58 ± 33.15	3+
Pectin	24.25 ± 0.53^{b}	126.74 ± 3.68	2+	22.94 ± 2.92^{a}	117.62 ± 20.26	2+
Chitin	24.06 ± 0.80^{b}	125.43 ± 5.52	1+	32.25 ± 3.01^{a}	182.29 ± 20.87	1+
Sorbose	6.19 ± 0.09^{bc}	1.30 ± 0.60	1+	17.00 ± 3.01^{a}	76.39 ± 20.83	1+
Glucose	14.38 ± 1.94^{a}	58.16 ± 3.50	3+ ~ 4+	29.53 ± 4.23^{a}	163.37 ± 37.14	3+ ~ 4+

4+=dense; 3+=moderate; 2+scanty; 1+=very scanty. Values are expressed as means ± SD of triplicates. Means followed by the same superscript indicate no significant difference at the level of p=0.05.

Table 4. Growth of Pf CM4 on various nitrogen sources

Nitrogen sources	Wild type			Pf CM4		
	Radial mycelia extension (*Rme*, mm)	Colony radial growth rate (*Kr*, μm.h^{-1})	Mycelia density	Radial mycelia extension (*Rme*, mm)	Colony radial growth rate (*Kr*, μm.h^{-1})	Mycelia density
Yeast Extract	34.31±1.68^{a}	196.61±11.66	5+	39.63±0.18^{a}	233.51±1.23	5+
Beef Extract	31.19±1.68^{a}	174.91±11.66	4+	39.19±1.86^{a}	230.00±12.89	5+
Tryptone	19.50±0.88^{c}	93.75±6.14	3+	37.00±1.77^{a}	215.28±12.28	4+
Peptone	15.69±2.21^{b}	67.27±15.35	2+	34.94±0.97^{a}	200.95±6.75	4+
Casaminoacids	20.38±2.28^{b}	99.83±19.63	2+	36.06±0.44^{a}	208.77±3.07	3+
L-asparagine	26.81±2.03^{b}	144.53±14.12	2+	38.75±0.88^{a}	227.43±6.14	2+～3+
NH_4Cl	20.06±3.62$^{ab/cd}$	97.66±25.17	2+	35.38±0.71^{a}	203.99±4.91	3+
NH_4NO_3	15.06±2.92^{ab}	62.93±20.26	1+	37.38±0.35^{a}	217.88±2.46	2+
$NaNO_3$	17.38±1.59^{ab}	78.99±11.05	1+	28.75±2.47^{b}	157.99±17.19	1+
Urea	27.75±1.41^{cd}	76.39±15.96	2+	35.13±0.71^{a}	202.29±4.91	2+
$(NH_4)_2SO_4$	17.00±2.30^{ab}	144.53±14.12	1+	36.75±0.35^{a}	213.54±2.46	2+

5+=very dense; 4+=dense; 3+=moderate; 2+scanty; 1+very scanty. Values are expressed as means±SD of triplicates. Means followed by the same superscript indicate no significant difference at the level of p=0.05.

growths of the wild type and Pf CM4 were significantly influenced by the incubation temperature ($p < 0.05$), and it was found that Pf CM4 preferred a high temperature (30˚C), while the wild type grew well at 25˚C (Fig. 1). The lowest mycelia growth was recorded at 16˚C for both Pf CM4 and the wild type. The lowest growth at low temperature may be due to the reduced metabolic activities necessary for the absorption of the essential nutrients required for growth (Garraway and Evans, 1984). According to Shim *et al.* (2003), the mycelia growth is expedited gradually in proportion to the increase in temperature. The same trend was observed in the present study with Pf CM4, but the mycelia growth was suppressed at 32˚C. It was observed that the level of mycelia growth suppression at 32˚C was not as low as that found at 25˚C ($p < 0.01$). The optimum temperature for the mycelia growth of *P. ostreatus* was 25˚C (Adebayo-Tayo *et al.* 2011). Imtiaj *et al.* (2009) observed that the mycelia growth of *Paecilomyces fumosoroseus* was suppressed at temperatures higher than 30˚C. Imtiaj *et al.* (2007) reported that 25˚C was the most suitable temperature for the spontaneous mycelia growth of the majority of mushrooms, but our findings obtained using the mutant were not in agreement with this result. This could be correlated with the existence of genetic variations, resulting from an induced radiation mutagenesis, in the tolerance to high temperature. Chen *et al.* (2008) developed a high temperature tolerant *P. ferulae* using mutagenesis through nitrogen ion implantation. In the present study, it should be noted that the mutant had the ability to grow even at high temperature compared with the wild type ($p < 0.05$). This characteristic appears to be a need of the hour for sustainable mushroom farming, mainly in tropical regions.

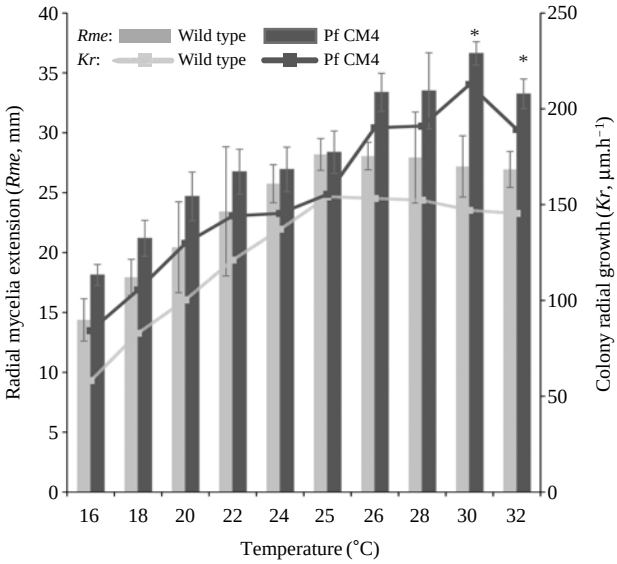

Fig. 1. Incubation temperature influenced the mycelia growth of the wild type and Pf CM4. *Pf CM4 recorded significantly high growth compared with the wild type ($p < 0.05$).

It was observed that all pH levels tested (5.0 to 9.0) favoured the growth of Pf CM4 and the wild type, and no significant difference in mycelia growth was found between pH 5.0 and 9.0 ($p > 0.05$). However, the optimal pH for the maximal mycelia growth of Pf CM4 and the wild type was found to be 5.5 to 6.5, and 6.0 to 7.0, respectively, based on the maximum mean values (Fig. 2). Similar results were reported by Imtiaj *et al.* (2009), who studied 371 strains of mushrooms

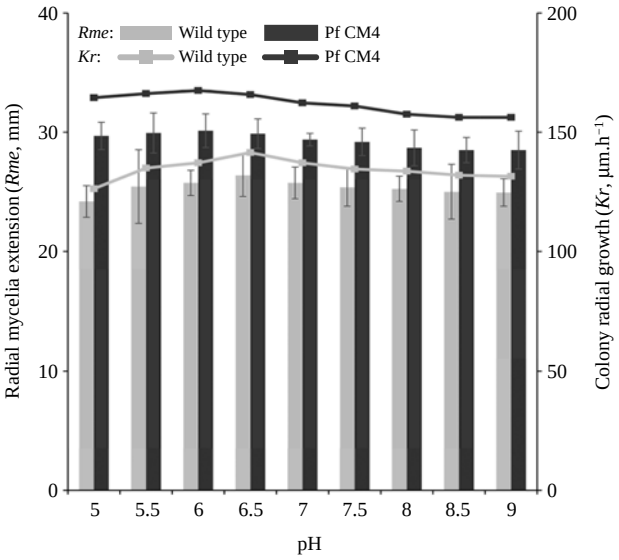

Fig. 2. Pf CM4 has a broad pH range for its mycelia growth similarly with that of the wild type. Significant difference in the mycelia growth was observed between Pf CM4 and the wild type ($p < 0.05$).

and observed that most of the mushrooms have a broad range of pH (5.0 ~ 9.0) for their mycelia growth, but the most favourable pH was between pH 6.0 and 7.0. In the case of Pf CM4, a slight decrease in growth at a basic pH was obtained ($p > 0.05$) compared with an acidic pH. This trend may be due to the sensitivity of a mutant to a basic pH compared with an acidic pH, as reported by Bilgrami and Verma (1992), who stated that mushroom mycelia are more tolerant to acidic media than to basic media. Ibekwe *et al.* (2008) observed that the optimum mycelia production of *P. ostreatus* was recorded at pH 6.5, while a pH range of less than 5.0 showed no significant growth. It seems that Pf CM4 has a broad pH range for its mycelia growth, similarly with that of the wild type, and that the irradiation might not have any effect on this trait.

CONCLUSION

The cellulolytic activity of *P. florida* could be significantly improved through gamma radiation induced mutagenesis. The mutant, Pf CM4, was found to be more potent (17.24%) than the wild type in terms of utilizing cellulose, in addition to various polysaccharides. In particular, Pf CM4 could grow better at a high temperature than the wild type. Further studies will focus on the genomics and proteomics of this mutant for better employment in the recycling of cellulosic wastes and the sustainable production of edible mushrooms.

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