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Agro-industrial byproducts for production of the immunosuppressant mycophenolic acid by *Penicillium roqueforti* under solid-state fermentation: Enhanced production by ultraviolet and gamma irradiation

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ABSTRACT

Different agro-industrial materials were screened for their performance on production of mycophenolic acid (MPA) by two strains of *Penicillium roqueforti* (AG101 and LG109) under solid-state fermentation (SSF). Sugarcane bagasse followed by corn stalks and rice bran were the most favorable substrates supporting the MPA production. The maximum MPA concentration was achieved at initial moisture content 70% for the first substrate and 80% for the other substrates. Enrichment of the solid substrate with a modified Czapek's broth promoted a greater MPA concentration than that enriched with mineral salt solution. The AG101 and LG109 strains were improved to produce higher significant MPA titers on solid substrate by subjecting the spores to irradiation by UV light (254 nm) for 120 and 90 min, respectively; and gamma rays at 0.75 KGy. The highest MPA-producing ability of both irradiated strains was observed on sugarcane bagasse and it was increased by 1.57–1.72 fold when irradiated with UV rays and by 1.74–1.92 fold when irradiated with gamma rays, as compared with the non-irradiated cultures. These findings indicate the future possibility to reduce the cost of producing fermentation-based drugs.

1. Introduction

Over the years, a lot of effort has been devoted to reduce the manufacturing costs of drugs. Several fungal fermentation processes are commonly used in pharmaceutical industry for large-scale production of drugs. Among the fermentation-based drugs, mycophenolic acid (6-(4-hydroxy-6-methoxy-7-methyl-3-oxophthalanyl)-4-methyl-4-hexenic acid) (MPA) is one of the most commonly prescribed immunosuppressants. MPA and its derivatives such as mycophenolate mofetil and sodium mycophenolate are applicable in treatment of patients with organ transplantation and autoimmune diseases owing to their inhibitory effect on inosine monophosphate dehydrogenase of proliferating B and T lymphocytes. This leads to cessation of the purine pathway and thereby decreases the titer of these immune cells and attenuates the rejection mechanism (Mele and Halloran, 2000). Thus, MPA and its derivatives seem to be the main alternatives for replacement of drugs already in use as immunosuppressants (e.g. cyclophosphamide, methotrexate, azathioprine, and cyclosporin A) which had serious problems such as toxicity, lack of reversibility and increased susceptibility to viral and other infections (Vinokurova et al., 2005). MPA was also reported to exhibit diverse biological properties including antiviral (Diamond et al., 2002), antifungal (Nicoletti et al., 2004), antibacterial (Torrenegra et al., 2005), antitumor (Tressler et al., 1994), and antipsoriasis activities (Epinette et al., 1987).

On the basis of the broad clinical utility of MPA, some attempts have been made on the production of MPA by submerged and solid-state batch cultures of *Penicillium brevicompactum* (Alani et al., 2009; Shu et al., 2010; Patel et al., 2017, 2018). MPA was also identified in extracts of some strains of *P. roqueforti* but in a lower concentration than that synthesized by *P. brevicompactum* (Lafont et al., 1979). Recently, a genomic region of approximately 24.4 kbp containing a seven-gene cluster (the *mpa* cluster) that may be involved in the MPA biosynthesis in *P. roqueforti* was identified (Del-Cid A et al., 2016). Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all commercial fermentation processes. Modification and improvement of the strain through mutation are typically achieved by subjecting the genetic material to physical or chemical mutagens (Parekh et al., 2000). Among physical mutagens, UV rays have medium effect, which induces pyrimidine dimerization by frame shift transition from GC to AT base pair (Chopra, 2005). Gamma rays (the most

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energetic ionizing radiation) cause mutations as single or double strand breakage of DNA by deletion or structural change, DNA-protein cross links, oxidized bases and basic sites (Cadet et al., 1999).

Solid-state fermentation (SSF) involves the growth of microorganism on moist solid substrate in the absence of free-flowing water. It has been applied successfully for production of cyclosporin A by *Tolypocladium inflatum* (Ramana Murthy et al., 1999), penicillin by *P. chrysogenum* (Barrios-González et al., 1988), red and yellow pigments by *Monascus purpurea* (Chiu and Chan, 1992), and citric acid by *Aspergillus niger* (Kumar et al., 2003). However, information and experience regarding the production of MPA by *P. roqueforti* via fermentation are very limited. Nevertheless, only two studies in literature focused on MPA production from *P. brevicompactum* by SSF. First, moist wheat bran supplemented with nutrients was used in order to maximize the MPA titre (Sadhukhan et al., 1999). Second, the superiority of SSF to SmF in flask cultures was reported and the potential scale-up of the SSF in a packed-bed bioreactor using pearl barley as a solid substrate was explored (Alani et al., 2009). Thus, developing a cost-effective MPA fermentation process becomes essential. Our success in achieving high titres of MPA from UV and gamma irradiated *P. roqueforti* strains by SmF (Ismaiel et al., 2014), and immobilization (Ismaiel et al., 2015), encouraged us to explore the possibility of SSF for production of MPA by these irradiated strains. In this paper, we screened different agro-industrial wastes for MPA production from two strains of *P. roqueforti* by SSF. Additionally, further improvement in the MPA production of the solid-state grown cultures was adopted by UV and gamma irradiation.

2. Materials and methods

2.1. Fungal strains

Penicillium roqueforti AG101 and LG109 were identified and selected among 30 strains isolated from Roquefort cheese samples on the basis of their activity for MPA-producing ability from our preliminary screening studies (Ismaiel et al., 2014). Cultures were maintained on yeast-sucrose (YES) agar slants and stored at 7 °C.

2.2. Inoculum preparation

Fungal spores from 5-day old cultures of both strains were harvested separately by flooding of the slants with sterile water containing 0.1% Tween 20 and gently scrapping off the spores with a sterile glass rod. The spore concentration was adjusted to 2×10^6 spores ml^{-1} using a haemocytometer (Ismaiel et al., 2014).

2.3. Agro-industrial materials

A total of six different by-products (sugarcane bagasse, corn stalks, rice bran, wheat bran, rice husk and wheat husk) were used as solid substrates for testing the ability of the two strains to produce MPA. Sugarcane bagasse was kindly provided by a local sugarcane mill (Menia El-Kamh, Egypt) and other substrates were purchased from local stores. These materials were oven-dried at 60 °C, then milled to 0.2- to 0.5- mm particle size.

2.4. MPA production by solid-state fermented fungal cultures

Ten gram samples of solid substrates were separately placed in 250 ml Erlenmeyer flasks and varying amounts of mineral salt solution (MSS) (composition, g l^{-1}: $MgSO_4.7H_2O$, 0.05; KCl, 0.05; $FeSO_4.7H_2O$, 0.01) were added as moistening agent to get the desired different moisture contents at 60, 70, 80 and 90%. The flasks were then autoclaved for 30 min at 121 °C. After cooling the flasks to room temperature, 3 ml of 5-day old spore suspension (2.0×10^6 spores ml^{-1}) either from AG101 or LG109 cultures was added and the contents of the flasks were thoroughly mixed, after which the flasks were incubated at 25 °C for 10 days. Meanwhile, SSF was also performed using semisynthetic modified broth (SSMB) as moistening agent (composition g l^{-1}: sucrose, 30; peptone, 5; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; $FeSO_4.7H_2O$, 0.01). The nutrients formulation and the required incubation conditions (at 25 °C for 10 days) were found necessary for the highest MPA production by the two *P. roqueforti* strains according to our previous optimization studies (Ismaiel et al., 2014). Details of SSF conditions were running as described earlier except replacing the MSS with the SSMB as moistening agent.

2.5. Effect of UV irradiation

Spore suspension of both AG101 and LG109 strain was prepared and adjusted to a concentration of 2.0×10^6 spores ml^{-1}, as described earlier. The freshly collected spore suspension of the first strain was exposed to UV light radiation (254 nm, 70 μw/cm^2) for 120 min at a distance of 10 cm from the UV source and that of the second strain was exposed to the same UV source for 90 min at the same distance. The UV exposure conditions of the two strains at the doses applied were performed according to our preliminary results concerning enhanced production of MPA (Ismaiel et al., 2014). Based on these results, spore viability of both UV-irradiated AG101 and LG109 strains was 4.10 and 5.70%, respectively. The UV exposed spore suspensions were stored in dark overnight at 4 °C (to avoid photo-reactivation) then 3 ml aliquot was added separately to 250 ml Erlenmeyer flasks containing sterilized solid waste–MSS mixture. The agricultural wastes tried with their moisture contents were selected on the basis of the availability for MPA production. These wastes were sugarcane bagasse (at initial moisture content of 70%), corn stalks (at initial moisture content of 80%) and rice bran (at initial moisture content of 80%). Another portion of the UV irradiated spore suspension (3 ml) either of AG101 or LG109 strain was also added to 250 ml Erlenmeyer flasks containing sterilized solid waste–SSMB mixture adjusted at the favorable moisture contents for MPA production. Flasks were incubated at 25 °C for 10 days as static cultures and evaluated for the production of MPA.

2.6. Effect of gamma irradiation

Irradiation process was carried out at Nuclear Research Center, Cairo, Egypt. The facility used was ^{60}Co Gamma chamber (MC20, Russia). The average dose rate of this gamma radiation source was 1.3 kGy h^{-1} at the time of the experiment. The freshly prepared spore suspensions (2.0×10^6 spores ml^{-1}) either of AG101 or LG109 strain were irradiated at 0.75 KGy which was found to be the most effective dose for achieving the highest MPA productivity by the two fungal strains (Ismaiel et al., 2014). At this dose, gamma irradiation showed approximately a survival of 40% of both strains. Gamma exposed spore suspensions were stored in dark overnight at 4 °C (to avoid photo-reactivation) then 3 ml aliquot of the irradiated spore suspension of AG101 and LG109 strain was added separately to 250 ml Erlenmeyer flasks containing agriculture waste either enriched with MSS or SSMB. The agriculture wastes tested were maintained at specific moisture contents as running in the UV irradiation steps and incubation was carried out under the optimum conditions for the production process (at 25 °C for 10 days).

2.7. Analytical methods

2.7.1. Determination of dry biomass

The cell mass and the residual substrate were separated and estimated at the end of incubation period as per method reported (Augustine et al., 2006). The fermented substrate was homogenized and triplicate samples of 1.0 g from each flask were transferred to pre-weighed centrifuge tubes and 5 ml of sodium sulphate (150 g l^{-1}) was added to each tube. The tubes were centrifuged at $12000 \times g$ for 15 min.

Centrifugation was repeated thrice to achieve complete separation of fungal mass (floated) from the substrate (settled to the bottom). The biomass alone was transferred to a pre-weighed filter paper and oven dried at 80 °C to a constant weight.

2.7.2. Extraction of MPA from P. roqueforti cultures

The fermented matter was thoroughly mixed with distilled water (1: 5, w/v) on a rotary shaker at 200 rpm for 1 h. After which, MPA was extracted from the resulting supernatant according to Puel et al. (2005) with some modifications. The supernatant of the two fungal strains were adjusted separately to pH 2.0 with 2M H_2SO_4, and defatted with *n*-hexane after which MPA was extracted from the culture filtrate with an equal volume of ethyl acetate for 1 h. The ethyl acetate layer was filtered over anhydrous sodium sulfate and then evaporated under vacuum until dryness using rotary evaporator (IKA, RV10, Germany). The dried crude extract was dissolved in methanol and undergone the necessary chromatographic analysis.

2.7.3. Qualitative and quantitative analysis of MPA

The methanol extract samples were loaded with reference standard of MPA (Applichem GmbH, Darmstadt, Germany) on TLC plates using toluene: ethyl acetate: formic acid (6:3:1, v:v:v) as a developing system. MPA spots (Rf = 0.65) show blue influorescence under long-wave length UV-light using Min UVIS, DUOUV source for TLC. The spots were more visualized by exposing of the chromatograms to ammonia fumes before observation under UV-light. The fluorescent bands corresponding to the authentic MPA were scrapped off and eluted with chloroform and further rechromatographed. MPA was then quantified by ultraviolet spectroscopic analyses performed with a JENWAY-305 spectrophotometer, UK. MPA absorption was monitored at 304 nm, and the concentration was obtained after recording the optical density against a standard curve (Queener and Nash, 1978).

The identity of MPA was also confirmed by high performance liquid chromatography (HPLC, EZChrom Elite Client/Server, Agilent, USA). The HPLC analysis column specifications are 4.5 × 250 nm, 2.5 µm of Hypersil BDS-C18 at 40 °C. The mobile phase consisted of methanol and 0.01% phosphoric acid at a ratio of 80:20, pH 3.0 and was used at a flow rate of 1.0 ml min^{-1}. The UV detector was set at a wavelength of 254 nm and an injection volume of 20 µl was used (Shu et al., 2010). The data of HPLC analysis of the sample extracted (either from AG101 or LG109 strain) were identical with those of the authentic sample of MPA analyzed under the same conditions (Fig. 1).

2.8. Statistical analyses

Results were expressed as the mean ± standard deviation (SD). Statistical significance was evaluated using analysis of variance (ANOVA, SPSS software version 22, IBM Corp., NY) test followed by the least significant difference test at 0.05 level.

3. Results

3.1. Suitability of different solid substrates moistened with MSS for MPA production

Different agro-industrial materials as solid substrates were screened for MPA production by *P. roqueforti* AG101 and LG109 (Table 1). Sugarcane bagasse was found to support the maximum rates of both growth and MPA, followed by corn stalks, rice bran and wheat bran. Meanwhile, wheat husk and rice husk achieved the lowest rates of MPA productivities by the two fungal strains (Table 1). These results indicated that MPA productivity was found to be greatly affected by the type of the employed substrate.

Data presented in Table 1 further demonstrated that the production of MPA using different solid substrates was also affected by the moisture content. The initial moisture content of 70% was found to support maximum production of MPA (7.59 ± 0.46 mg kg^{-1} and 7.31 ± 0.52 mg kg^{-1} by AG101 and LG109 strain, respectively) and growth (0.642 ± 0.03 g g^{-1} and 0.652 ± 0.02 g g^{-1}, respectively), on using sugarcane bagasse as a cultural substrate. However, a further increase in the moisture content to 80 and 90% showed a significant decrease in both growth and MPA production by the two fungal strains. The obtained data also showed that MPA production increased with the increase in the moisture content until it reached maximum at 80%, on using corn stalks, rice bran, wheat bran, rice husk and wheat husk as solid substrates. It is interesting to mention that growth and MPA production by the two fungal strains on solid substrates are proportionate process, where the MPA production increased with the increase in the dry cell weights and vice versa.

3.2. Effect of UV irradiation on MPA production by SSF using MSS as a moistening agent

Fig. 2 illustrated the effect of exposure to UV irradiation on cell growth and MPA production of both strains grown under SSF, as compared with the control treatments (without UV exposure). At all the tried solid substrates, the dry cell weight of the UV-irradiated AG101 and LG109 strain was reduced when compared with control treatments. However, MPA production under SSF was significantly ($P \le 0.05$) enhanced following irradiating the spores of AG101 and LG109 cultures with UV light. When sugarcane bagasse was used as a solid substrate, the dry cell weight of the UV-irradiated AG101 and LG109 cultures was reduced to 0.551 ± 0.020 g g^{-1} and 0.557 ± 0.021 g g^{-1}, respectively. However, the MPA concentrations produced by UV-irradiated cultures of AG101 and LG109 strain reached 8.98 ± 0.03 mg kg^{-1} and 8.46 ± 0.02 mg kg^{-1}, respectively. In the case of using corn stalks as solid substrate, MPA concentrations production by UV-irradiated AG101 and LG109 cultures increased to 8.41 ± 0.02 mg kg^{-1} and 7.55 ± 0.03 mg kg^{-1}, respectively. Moreover, the MPA concentration from irradiated AG101 and LG109 cultures reached 8.11 ± 0.07 mg kg^{-1} and 7.82 ± 0.03 mg kg^{-1}, respectively in case

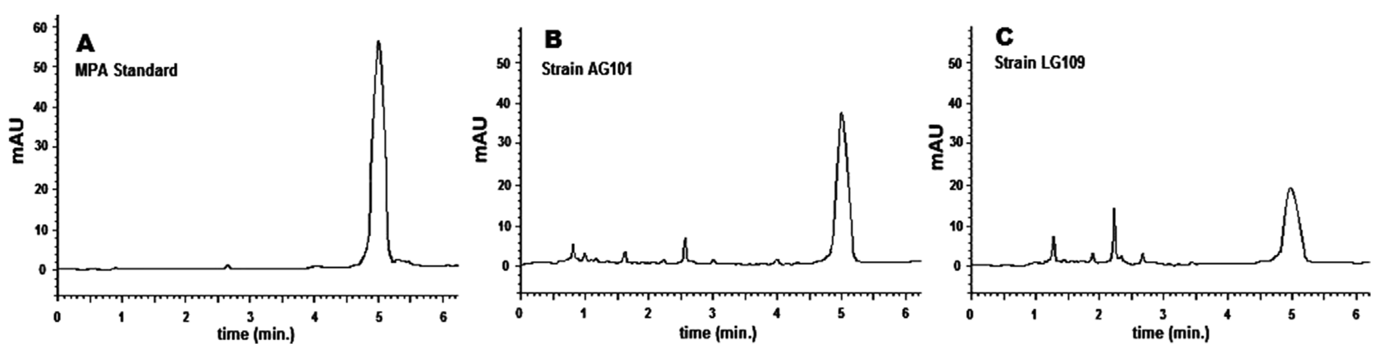

Fig. 1. The HPLC chromatograms of MPA standard (A) and samples obtained by both AG101 (B) and LG109 (C) strain.

Table 1
Dry cell weights (g g^{-1} dry substrate) and MPA concentration (mg kg^{-1} dry substrate) of *P. roqueforti* AG101 and LG109 strain grown on different wastes moistened with MSS at different moisture contents (%).

Wastes	Moisture content (%)	*P. roqueforti* AG101		*P. roqueforti* LG109	
		Dry cell weights (g g^{-1})	MPA conc. (mg kg^{-1})	Dry cell weights (g g^{-1})	MPA conc. (mg kg^{-1})
Corn stalks	60	0.318 ± 0.01^{c}	3.67 ± 0.10^{d}	0.323 ± 0.02^{b}	3.58 ± 0.16^{d}
	70	0.480 ± 0.02^{ab}	4.87 ± 0.36^{c}	0.413 ± 0.01^{b}	4.74 ± 0.03^{c}
	80	0.553 ± 0.02^{a}	6.33 ± 0.03^{a}	0.580 ± 0.02^{a}	6.12 ± 0.05^{a}
	90	0.426 ± 0.05^{b}	5.45 ± 0.04^{b}	0.454 ± 0.02^{b}	5.01 ± 0.04^{b}
Rice bran	60	0.301 ± 0.03^{c}	3.62 ± 0.18^{2}	0.324 ± 0.05^{c}	3.48 ± 0.18^{c}
	70	0.433 ± 0.03^{b}	4.75 ± 0.03^{c}	0.464 ± 0.02^{ab}	4.69 ± 0.08^{b}
	80	0.511 ± 0.02^{a}	5.98 ± 0.06^{a}	0.545 ± 0.02^{a}	5.53 ± 0.06^{a}
	90	0.396 ± 0.02^{b}	5.39 ± 0.11^{b}	0.399 ± 0.01^{bc}	5.14 ± 0.20^{ab}
Rice husk	60	0.287 ± 0.04^{b}	2.88 ± 0.09^{b}	0.295 ± 0.02^{b}	2.67 ± 0.02^{d}
	70	0.301 ± 0.02^{b}	3.42 ± 0.16^{ab}	0.327 ± 0.01^{b}	3.39 ± 0.07^{c}
	80	0.390 ± 0.01^{a}	4.73 ± 0.02^{a}	0.397 ± 0.03^{a}	4.68 ± 0.03^{a}
	90	0.316 ± 0.03^{b}	4.24 ± 1.17^{ab}	0.333 ± 0.02^{b}	4.05 ± 0.05^{b}
Sugarcane bagasse	60	0.431 ± 0.01^{c}	5.88 ± 0.06^{c}	0.448 ± 0.01^{c}	5.58 ± 0.06^{b}
	70	0.642 ± 0.03^{a}	7.59 ± 0.46^{a}	0.652 ± 0.02^{a}	7.31 ± 0.52^{a}
	80	0.587 ± 0.01^{b}	6.52 ± 0.04^{b}	0.596 ± 0.01^{b}	6.16 ± 0.09^{b}
	90	0.453 ± 0.01^{c}	5.98 ± 0.01^{c}	0.466 ± 0.02^{c}	5.61 ± 0.11^{b}
Wheat bran	60	0.203 ± 0.01^{c}	3.41 ± 0.12^{d}	0.219 ± 0.07^{c}	3.36 ± 0.06^{c}
	70	0.323 ± 0.03^{b}	3.89 ± 0.09^{c}	0.333 ± 0.04^{b}	3.57 ± 0.11^{bc}
	80	0.404 ± 0.07^{a}	4.96 ± 0.07^{a}	0.427 ± 0.02^{a}	4.79 ± 0.04^{a}
	90	0.347 ± 0.02^{b}	4.19 ± 0.0^{6b}	0.363 ± 0.03^{ab}	4.06 ± 0.50^{b}
Wheat husk	60	0.289 ± 0.03^{c}	2.82 ± 0.12^{d}	0.293 ± 0.03^{b}	2.78 ± 0.10^{d}
	70	0.321 ± 0.02^{bc}	3.13 ± 0.11^{c}	0.346 ± 0.02^{a}	3.04 ± 0.05^{c}
	80	0.383 ± 0.02^{a}	4.42 ± 0.04^{a}	0.393 ± 0.02^{a}	4.39 ± 0.15^{a}
	90	0.347 ± 0.02^{ab}	4.13 ± 0.03^{b}	0.368 ± 0.01^{a}	4.09 ± 0.06^{b}

Solid–state grown cultures were carried out at 25 °C for 10 days, moistened with MSS containing an inoculum size 3 ml/250 ml flask, calculated mean is for triplicate measurements from two independent experiments ± SD, $^{a-d}$means with different superscripts in the same column for each individual substrate are considered statistically different (LSD test, $P \le 0.05$).

of using rice bran.

3.3. Effect of gamma irradiation on MPA production by SSF using MSS as a moistening agent

Gamma irradiation appears to have a greater effect than UV irradiation on both dry cell weight and MPA production by the solid state-grown AG101 and LG109 cultures (Fig. 3). On using sugarcane bagasse, corn stalks and rice bran as solid substrates, the dry cell weight of gamma-irradiated AG101 cultures was reduced by 31.5%, 42.1% and 23.1%, respectively; as compared with their respective control cultures. A similar reducing effect on dry cell weight of the second strain grown on the respective substrates was also obtained. The dry cell weight was reduced by 28.4%, 39.7% and 26.8%, respectively; as compared with control cultures. However, a significant enhancement ($P \le 0.05$) in MPA concentrations produced by gamma-irradiated AG101 and LG109 cultures as compared with control cultures (non-irradiated). Gamma irradiation induced significant enhancement in MPA concentration of AG101 strain on using sugarcane bagasse, corn stalks and rice bran as solid substrates. MPA concentrations were 1.51, 1.70 and 1.72 fold of control cultures. On using the respective solid substrates, MPA concentrations produced by LG109 culture were 1.58, 1.66 and 1.82 fold of

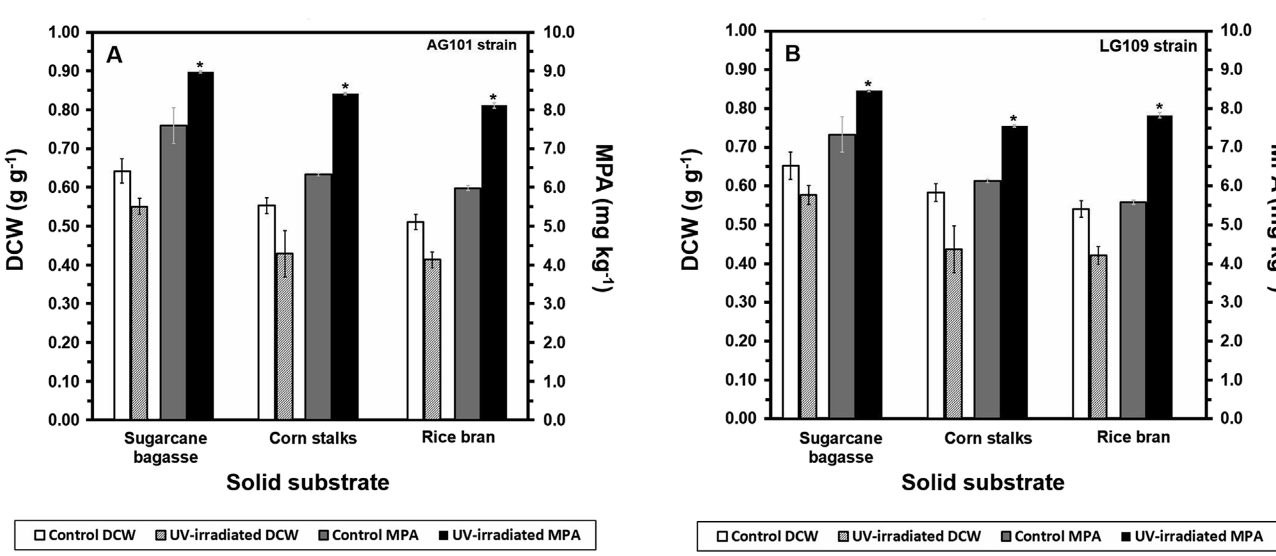

Fig. 2. Effect of UV-irradiation on growth (g g^{-1} dry substrate) and MPA production (mg kg^{-1} dry substrate) by solid–state grown culture of AG101 (A) and LG109 (B) strain. The solid substrates were moistened with MSS to get initial moisture content of 70% with sugarcane bagasse and 80% with corn stalks and rice bran. Data are shown as the mean ± SD of triplicate measurements from two independent experiments. *Significantly different from control (LSD test, $P \le 0.05$).

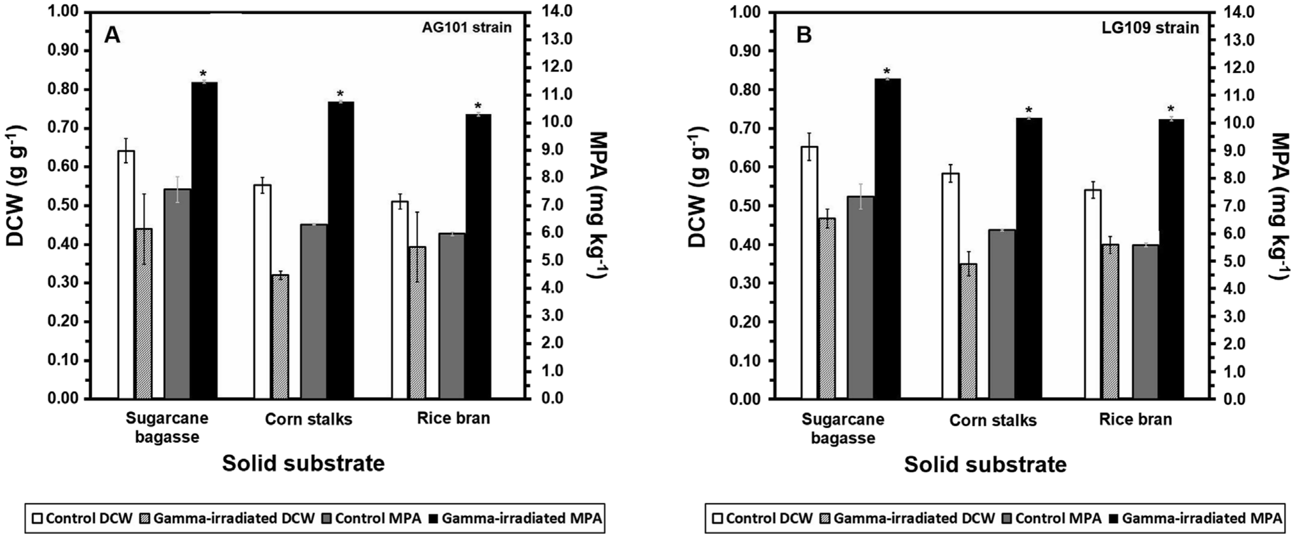

Fig. 3. Effect of gamma-irradiation on growth (g g^{-1} dry substrate) and MPA production (mg kg^{-1} dry substrate) by solid–state grown culture of AG101 (A) and LG109 (B) strain. The solid substrates were moistened with MSS to give moisture content of 70% with sugarcane bagasse and 80% with corn stalks and rice bran. Data are shown as the mean ± SD of triplicate measurements from two independent experiments. *Significantly different from control (LSD test, $P \le 0.05$).

control cultures.

3.4. Suitability of different solid substrates moistened with SSMB for MPA production

Table 2 demonstrated the effect of applying SSMB as a moistening agent on growth and MPA production by the two strains. Sugarcane bagasse was the most favorable solid substrate that supported maximum levels of both cell growth and MPA production by the two *P. roqueforti* strains. At 70% initial moisture content of sugarcane bagasse, cell growth (0.987 ± 0.015 g g^{-1} of AG101 strain and 0.995 ± 0.011 g g^{-1} of LG109 strain) and MPA production (9.42 ± 0.03 mg kg^{-1} by the first strain and 8.65 ± 0.08 mg kg^{-1} by the second strain) were maximum. Moreover, at 80% moisture content, corn stalks followed by rice bran were also good substrates for growth and MPA production. MPA production by AG101 and LG109 strain on corn stalks was 1.58 and 1.48 fold of that produced on wheat husk, respectively. On rice bran, MPA production was 1.43 and 1.37 fold of that produced on wheat husk by the two respective fungal strains. When rice husk and wheat bran were used as cultural substrates at an initial moisture content of 80%, both of cell growth (0.820–0.841 g g^{-1} for AG101 strain and 0.830–0.868 g g^{-1} for LG109 strain) and MPA

Table 2
Dry cell weights (g g-1 dry substrate) and MPA concentration (mg kg^{-1} dry substrate) of *P. roqueforti* AG101 and LG109 strain grown on different wastes moistened with the SSMB at different moisture contents (%).

Wastes	Moisture content (%)	*P. roqueforti* AG101		*P. roqueforti* LG109	
		Dry cell weights (g g^{-1})	MPA conc. (mg kg^{-1})	Dry cell weights (g g^{-1})	MPA conc. (mg kg^{-1})
Corn stalks	60	0.743 ± 0.09^{b}	5.25 ± 0.32^{d}	0.752 ± 0.09^{a}	4.85 ± 0.12^{d}
	70	0.807 ± 0.02^{ab}	6.73 ± 0.06^{c}	0.833 ± 0.06^{a}	5.85 ± 0.03^{c}
	80	0.879 ± 0.02^{a}	8.80 ± 0.02^{a}	0.897 ± 0.01^{a}	7.83 ± 0.01^{a}
	90	0.857 ± 0.06^{ab}	7.99 ± 0.01^{b}	0.870 ± 0.09^{a}	6.94 ± 0.16^{b}
Rice bran	60	0.724 ± 0.06^{b}	4.43 ± 0.04^{d}	0.736 ± 0.05^{b}	4.37 ± 0.09^{d}
	70	0.843 ± 0.08^{a}	5.36 ± 0.07^{c}	0.870 ± 0.02^{a}	5.59 ± 0.03^{c}
	80	0.868 ± 0.07^{a}	7.96 ± 0.04^{a}	0.876 ± 0.027^{a}	7.26 ± 0.22^{a}
	90	0.819 ± 0.09^{a}	7.86 ± 0.01^{b}	0.836 ± 0.07^{a}	6.77 ± 0.05^{b}
Rice husk	60	0.703 ± 0.03^{b}	3.21 ± 0.39^{d}	0.721 ± 0.03^{b}	3.05 ± 0.15^{d}
	70	0.807 ± 0.06^{a}	4.91 ± 0.10^{c}	0.816 ± 0.02^{ab}	4.74 ± 0.21^{c}
	80	0.820 ± 0.09^{a}	6.61 ± 0.04^{a}	0.830 ± 0.09^{a}	6.46 ± 0.17^{a}
	90	0.743 ± 0.01^{ab}	6.16 ± 0.04^{b}	0.753 ± 0.03^{ab}	6.05 ± 0.02^{b}
Sugarcane bagasse	60	0.770 ± 0.01^{b}	6.54 ± 0.07^{c}	0.783 ± 0.07^{b}	5.63 ± 0.04^{c}
	70	0.987 ± 0.02^{a}	9.42 ± 0.03^{a}	0.995 ± 0.01^{a}	8.65 ± 0.08^{a}
	80	0.910 ± 0.02^{a}	7.31 ± 0.49^{b}	0.936 ± 0.02^{a}	6.37 ± 0.06^{b}
	90	0.888 ± 0.07^{ab}	6.69 ± 0.08^{c}	0.896 ± 0.02^{ab}	5.86 ± 0.13^{c}
Wheat bran	60	0.717 ± 0.02^{c}	4.16 ± 0.32^{d}	0.733 ± 0.05^{c}	4.05 ± 0.44^{d}
	70	0.814 ± 0.02^{ab}	5.12 ± 0.26^{c}	0.832 ± 0.03^{a}	5.09 ± 0.19^{c}
	80	0.841 ± 0.05^{a}	6.96 ± 0.12^{a}	0.868 ± 0.01^{a}	6.90 ± 0.06^{a}
	90	0.763 ± 0.06^{b}	6.72 ± 0.03^{b}	0.781 ± 0.08^{b}	6.12 ± 0.14^{b}
Wheat husk	60	0.657 ± 0.01^{c}	3.11 ± 0.38^{c}	0.672 ± 0.03^{c}	3.06 ± 0.16^{c}
	70	0.701 ± 0.01^{b}	4.83 ± 0.06^{b}	0.738 ± 0.02^{b}	4.41 ± 0.25^{b}
	80	0.796 ± 0.02^{a}	5.57 ± 0.26^{a}	0.810 ± 0.09^{a}	5.29 ± 0.10^{a}
	90	0.722 ± 0.03^{b}	5.09 ± 0.04^{b}	0.741 ± 0.01^{b}	4.83 ± 0.07^{ab}

Solid state-grown cultures were carried out at 25 °C for 10 days, moistened with SSMB containing an inoculum 3 ml/250 ml flask, calculated mean is for triplicate measurements from two independent experiments ± SD, $^{a\text{-}d}$means with different superscripts in the same column for each individual substrate are considered statistically different (LSD test, $P \le 0.05$).

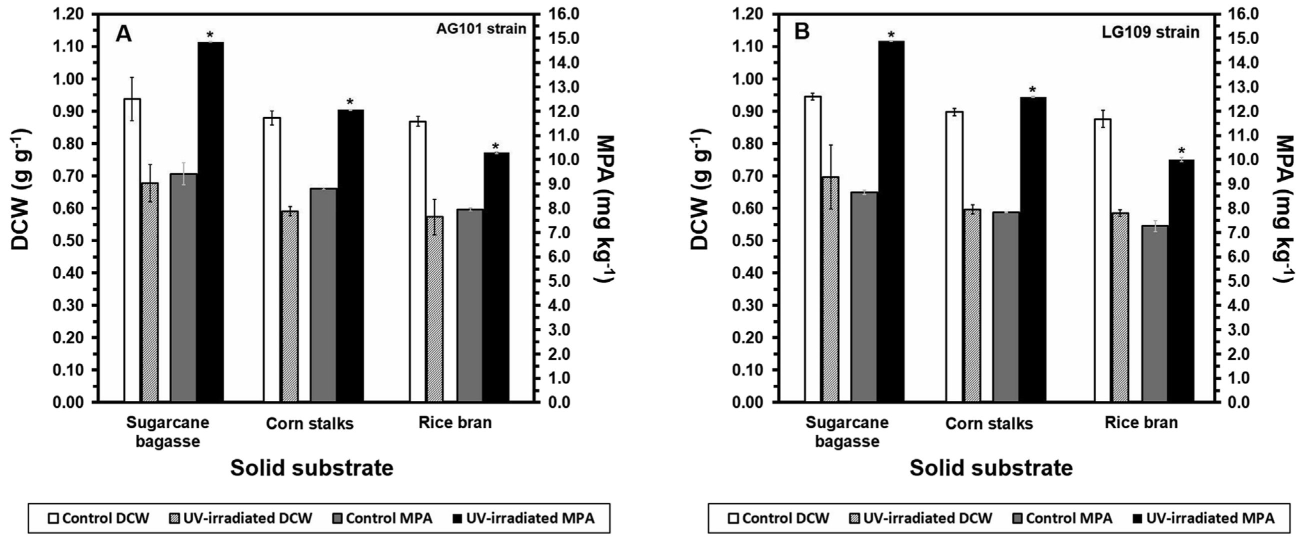

Fig. 4. Effect of UV-irradiation on growth (g g^{-1} dry substrate) and MPA production (mg kg^{-1} dry substrate) by solid–state grown culture of AG101 (A) and LG109 (B) strain. The solid substrates were moistened with SSMB to give moisture content of 70% with sugarcane bagasse and 80% with corn stalks and rice bran. Data are shown as the mean ± SD of triplicate measurements from two independent experiments. *Significantly different from control (LSD test, $P \leq 0.05$).

production (6.61–6.96 mg kg^{-1} and 6.46–6.90 mg kg^{-1}, respectively) reached similar levels (Table 2).

3.5. Effect of UV irradiation on MPA production by SSF using SSMB as a moistening agent

Data presented in Fig. 4 clearly indicated that growth and MPA production of both AG101 and LG109 strains under SSF were greatly affected by exposure to UV light. On using sugarcane bagasse, corn stalks and rice bran as cultural substrates, the dry cell weight of UV irradiated AG101 cultures was reduced approximately by 31.4%, 32.8% and 34%, as compared with their respective control cultures. The reduction in dry cell weight of UV irradiated LG109 cultures was approximately 30%, 33.4% and 33.2%, respectively. The production of MPA from solid state-grown *P. roqueforti* AG101 and LG109 cultures was significantly enhanced ($P \leq 0.05$) following irradiation with UV light, and the highest MPA concentration (14.85 ± 0.01 mg kg^{-1} and 14.89 ± 0.01 mg kg^{-1}) was observed on using sugarcane bagasse as a cultural substrate.

3.6. Effect of gamma irradiation on MPA production by SSF using SSMB as a moistening agent

Fig. 5 demonstrated that significant differences ($P \leq 0.05$) in MPA concentrations of gamma irradiated cultures either from AG101 or LG109 strain were obtained, as compared with control cultures. The highest MPA concentration by gamma-irradiated cultures (16.41 ± 0.11 mg kg^{-1} and 16.63 ± 0.11 mg kg^{-1}, respectively) was achieved on sugarcane bagasse as a solid substrate. In contrast, the dry cell weight of both gamma-irradiated fungal strains was reduced by 40.5% of that recorded at control treatments. On using corn stalks as solid substrates, the MPA concentration by gamma-irradiated cultures of AG101 and LG109 strain was increased significantly to 14.67 ± 0.041 mg kg^{-1} and 14.21 ± 0.053 mg kg^{-1}, respectively. However, the dry cell weight was reduced by 94.4% and 44.5%, respectively; of control cultures. In the case of using rice bran as solid substrate, the MPA concentration was also increased to 14.13 ± 0.132 mg kg^{-1} and 13.72 ± 0.232 mg kg^{-1}, respectively; while the dry cell weight was reduced by 48.5% and 46.8% of control treatments of the two respective fungal strains.

4. Discussion

Since filamentous fungi are often most suited to SSF for the production of valuable metabolites (Ramana Murthy et al., 1999; Sadhukhan et al., 1999), a total of six different agro-industrial materials were evaluated for their performance on production of MPA by the two *P. roqueforti* strains. When the solid substrates were moistened with MSS, a detectable variation of MPA titer was obtained regarding to the type of the employed substrate. The maximum rates of MPA production and growth of AG101 and LG109 strain were observed using sugarcane bagasse followed by corn stalks, rice bran and wheat bran. Otherwise, the lowest rates of MPA productivities by the two strains were measured in the presence of both wheat and rice husk as cultural substrates. The superiority of sugarcane bagasse over the other solid substrates in MPA production may be due to the difference in physical properties, particularly the smaller particle size or the availability of the nutrients. Corn stalks, rice bran and wheat bran were also good substrates for growth and MPA production. The differences in the protein and carbohydrate contents or the C/N ratio of the various substrates play a role in the production process (Alani et al., 2009). Sugarcane bagasse was reported to have lower protein content when compared to wheat and soy bean bran and its measured C/N ratio was 151.0, as compared to 16.9 and 5.4 for wheat and soy bean bran, respectively (Delabona et al., 2013).

The present study showed that the moisture content is a critical parameter in SSF for the production of high concentrations of MPA. Low moisture content reduces the solubility of nutrients provided to the organism by solid substrate, a lower degree of swelling and higher water tension, hence a less metabolic activity was resulted (Xavier et al., 1992). At higher moisture content, the inhibitory effect on fungal productivity was obtained due to the substrate particle agglomeration, lower O_2 transfer, and decreasing the porosity (Ramana Murthy et al., 1999). Generally, continuous gas phase prevails in the space between the solid particles and provides the dissolved oxygen to the microorganisms. Maximum water is absorbed in the moist solids on which the microbes grow. A thin water film is formed on the particles through which the gaseous oxygen passes to the microbes staying on the solid surface (Patel et al., 2017). SSF using bagasse with initial moisture content of 70% presented very high production rates of penicillin by *P. chrysogenum*, while the fermentation with lower initial moisture content (60%) presented fast antibiotic degradation rate (Barrios-González et al., 1988). A 70% initial moisture content was previously selected for

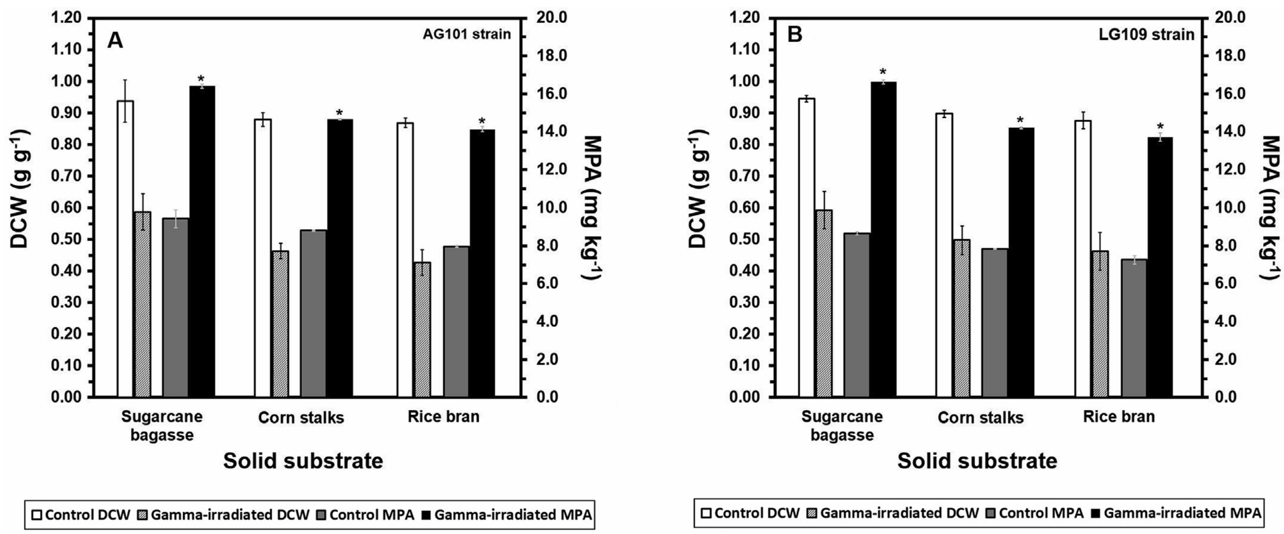

Fig. 5. Effect of gamma-irradiation on growth (g g^{-1} dry substrate) and MPA production (mg kg^{-1} dry substrate) by solid–state grown culture of AG101 (A) and LG109 (B) strain. The solid substrates were moistened with SSMB to give moisture content of 70% with sugarcane bagasse and 80% with corn stalks and rice bran. Data are shown as the mean ± SD of triplicate measurements from two independent experiments. *Significantly different from control (LSD test, P ≤ 0.05).

production of the immunosuppressants MPA (Alani et al., 2009; Sadhukhan et al., 1999), and cyclosporin A (Ramana Murthy et al., 1999).

Following UV exposure, the dry cell weight of AG101 and LG109 strain was reduced at all tried solid substrates, when compared with control treatments. For instance, on using sugarcane bagasse as a solid substrate the dry cell weight of the UV-irradiated AG101 and LG109 cultures was reduced by 14.3% and 11.5% of their respective control treatments (non-irradiated cultures). These results corroborate those obtained by Willocquet et al. (1996) who found that UV radiation effects as the number of exposition duration increased, indicating that both spore germination and mycelium growth were reduced, but not totally stopped by different exposures. In contrast, MPA production under SSF was significantly ($P \leq 0.05$) enhanced following irradiating the spores of AG101 and LG109 cultures to UV light. When sugarcane bagasse was used as a solid substrate, the MPA concentration produced by UV-irradiated cultures of AG101 and LG109 strain was 1.18 fold of their respective control cultures. On using corn stalks, the MPA enhancement by UV-irradiated cultures was 1.32 and 1.23 fold, respectively. On using rice bran, the MPA concentration increased to 1.36 and 1.42 fold, respectively; as compared with control cultures. The UV radiation has been recommended as a mutagen of first choice. UV rays are absorbed by pyrimidines (especially thymine) and induce pyrimidine dimerization by frame shift from GC to AT base pair (Chopra, 2005).

Gamma irradiation at 0.75 KGy showed a greater effect than UV irradiation on growth and MPA production by the solid state-grown AG101 and LG109 cultures. A remarkable reducing effect of gamma radiation on the dry cell weight of both strains was observed. Moreover, a significant increase in MPA concentrations produced from gamma-irradiated cultures when compared with control cultures (non-irradiated). The MPA concentrations from gamma irradiated AG101 culture strain grown on sugarcane bagasse, corn stalks and rice bran were 1.51, 1.70 and 1.72 fold of control cultures. The MPA concentrations by gamma irradiated LG109 culture strain grown on the respective solid substrates were 1.58, 1.66 and 1.82 fold of control cultures. This enhancement in MPA productivity by gamma rays may be attributed to the induction of some mutations to the genes of cells through the DNA repair mechanisms within the cells (Thacker, 1999). Similarly, Paster et al. (1985) found low mycelial growth of *A. ochraceus* following irradiation with 1.5 KGy, however an increase in ochratoxin production occurred from fungal cultures irradiated with 0.5, 1.0, 1.5, or 2.0 KGy. Moreover, El-Sayed et al. (2019) used UV and gamma irradiation at specific doses for production enhancement of the anticancer drug paclitaxel.

Supplementation of the solid substrates with the SSMB showed a remarkable effect on growth and MPA production by the two *P. roqueforti* strains. Amongst the solid substrates, sugarcane bagasse was found to be the most substrate supporting growth and MPA production by the two fungal strains. A satisfactory growth and MPA production was obtained also on corn stalks and rice bran impregnated with SSMB. With all solid substrates, the dry cell weight and MPA production increased gradually with the increase of moisture content reaching a maximum at 70% for sugarcane bagasse and 80% for the other solid substrates. Moreover, Patel et al. (2017) used adjusted the moisture content at 80% for maximum production of MPA by *P. brevicompacrum*. Again, this may be due to less metabolic activity at lower moisture contents (Xavier et al., 1992). However, at high moisture levels, aeration may be a problem due to reduced porosity (Ramana Murthy et al., 1999). Our results indicated that applying SSMB containing several nutrients as a moistening agent promoted a greater increase in MPA production by the two fungal strains than applying the mineral salt solution, MSS as a moistening agent. For instance, on addition of SSMB as a moistening agent to sugarcane bagasse (achieving an initial moisture content of 70%), the MPA concentration was intensified to 1.24 and 1.18 time by AG101 and LG109 strain, respectively; as compared with MPA concentration produced by sugarcane bagasse moistened with MSS at the same moisture content. These findings are consistent with those obtained by Sadhukhan et al. (1999) who found that the production of MPA from wheat bran increased from 425 to 3286 mg kg^{-1} after the addition of selected nutrients. In a previous study, the addition of $(NH_4)_2HPO_4$ (1%, w/w) as a nitrogen source to pearl barley had no effect on MPA production and even slightly decreased the MPA production to 5482 ± 186 mg kg^{-1} as compared with 5899 ± 367 mg kg^{-1} produced upon the addition of water alone (Alani et al., 2009). However, the addition of mannitol slightly increased the MPA production to 6390 ± 553 mg kg^{-1}. The same trend to our study was previously reported by Barrios-González et al. (1988) for high production of penicillin by *P. chrysogenum* grown on bagasse impregnated with culture medium with initial moisture content of 70%.

Production of MPA by the two fungal strains on the most favorable solid substrates moistened with SSMB at the optimum initial moisture content was manifested by irradiating the spore suspensions of both strains by UV rays. Additionally, the reducing effect of UV irradiation on cell growth of both strains grown on the tried solid substrates was remarkable when compared with control (non-irradiated strains). When sugarcane bagasse, corn stalks and rice bran were used as cultural substrates, the MPA of the UV irradiated AG101 culture was significantly enhanced to 1.57, 1.36 and 1.30 fold, respectively of their control treatments (non-irradiated cultures). On the same respective solid substrates, the MPA production enhancement by the UV irradiated LG109 culture was also significant recording 1.72, 1.64 and 1.38 fold of the control treatments. The mechanism of fungicidal activity of UV-C (200–280 nm) was well established and involved interaction of DNA through the absorption of photons (formation of photodimers between adjacent bases), preventing DNA replication and cell division (Becker et al., 1989).

It is interesting to mention that applying SSMB as a moistening agent is a critical parameter in SSF and is more effective for the production of high concentrations of MPA than applying MSS.

Gamma irradiation was more effective than UV irradiation in enhancing MPA concentrations by the two strains grown on solid substrates enriched with SSMB. The highest MPA production by both gamma irradiated AG101 strain and LG109 strain was recorded on sugarcane bagasse (with moisture content of 70%). In the case of corn stalks as solid substrate, the MPA concentration by gamma-irradiated cultures of AG101 and LG109 strain was increased significantly to 1.67 and 1.81 fold, respectively; of control cultures. On rice bran, the MPA concentration was also increased to 1.78 and 1.89 fold, respectively. With the three cultural substrates, a remarkable reduction in dry cell weight of gamma-irradiated AG101 and LG109 strain was observed as compared to control treatments (non-irradiated).

Several points can be drawn from this study. The production of MPA by the solid substrates tried may need enrichments with SSMB containing nutrients which have an essential role in promoting greater concentrations of MPA. Similarly, production of red and yellow pigments by *Monascus purpureus* was reported during cultivation of the fungus in wet bagasse containing PGY medium with corn oil in SSF (Chiu and Chan, 1992). Amongst the tried solid substrates, sugarcane bagasse was the most proper for MPA production by the two strains. Sugarcane bagasse has been successfully utilized as a substrate in SSF for production of valuable metabolites (Barrios-González et al., 1988; Kumar et al., 2003). Bagasse consists of approximately 50% cellulose and 25% each of hemicellulose and lignin. Chemically, bagasse contains about 50% α-cellulose, 30% pentosans and 2.4% ash. Because of its low ash content, bagasse offers numerous advantages for usage in bioconversion process using microbial cultures (Pandey et al., 2000). Gamma irradiation was more efficient than UV irradiation in further improvement of MPA production by *P. roqueforti* strains grown on solid substrates either humified with MSS or SSMB. This was clearly supported by our previous reports regarding MPA production enhancement following UV and gamma irradiation of submerged and immobilized cultures of *P. roqueforti* (Ismaiel et al., 2014, 2015). Comparing the MPA concentrations produced by gamma irradiated cultures of AG101 and LG109 strain under SSF in this work with those produced by the same gamma irradiated cultures under SmF in our previous work, we found that the concentration of MPA produced by SSF (using sugarcane bagasse as a cultural substrate) was approximately 2.8 times higher than MPA concentration produced by SmF (Ismaiel et al., 2014).

5. Conclusion

MPA production by *P. roqueforti* under SSF may need enrichments with nutrients which have an essential role in promoting a greater increase of MPA concentrations. The MPA-producing ability was more intensified by irradiating spores with UV and gamma irradiation. The highest concentration was obtained by gamma-irradiated cultures grown on sugarcane bagasse (16.41 ± 0.11 mg kg^{-1} and 16.63 ± 0.11 mg kg^{-1} by AG101 and LG109 strain, respectively) which was about 1.11 times higher than that obtained by UV-irradiated cultures. This study concluded the possibility of exploiting agricultural wastes by SSF for MPA production enhancement and this could be a good alternative approach to SmF. Further work should focus on identifying the hereditary stability and the mechanism by which UV and gamma-irradiation help cells to produce more MPA.

Conflicts of interest

The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.01.053.

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