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REGULAR ARTICLE

Effect of carbon, nitrogen and physico-chemical factors on patulin production in *Penicillium expansum*

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ABSTRACT

Environmental and nutritional factors play important roles in regulating production of mycotoxins. Few studies have been reported on the biosynthesis of patulin mycotoxin and the mechanisms that involve its biosynthesis in *Penicillium expansum*. Here, we investigated the effects of two culture media, pH, temperature, carbon and nitrogen sources and effect of carbon/nitrogen ratio on mycotoxin biosynthesis by *P. expansum* isolated from Moroccan cereals. It was found that pH and temperature had great influence on patulin production. Results also showed that carbon and nitrogen sources influenced patulin biosynthesis significantly in this strain. L-glutamate was optimized as important nitrogen source in synthetic culture medium. Effect of carbon/nitrogen ratio was evaluated which indicated the dependence of patulin production on this ratio. These results will provide useful information to better understand the regulatory mechanisms of patulin biosynthesis, and be helpful in developing effective means for controlling a mycotoxin contamination of foods and feeds.

1. Introduction

Mycotoxins are a relatively large and diverse group of naturally occurring fungal toxins, many of which are harmful to both humans and animals. They are secondary metabolites produced by some species of mould genera such as *Aspergillus*, *Penicillium* and *Fusarium* (Cast 2003), which enter the food chain in the field, after harvest (Alexa et al. 2012) during storage, or later in finished product under favorable conditions (Cast 2003, Alexa et al., 2012; Alborch et al., 2012). The main mycotoxins of importance in terms of health and food industry are aflatoxins (B1, B2, G1, G2), ochratoxin A, fumonisins, some trichothecenes, zearalenon, citrinin and patulin (Hussein et al., 2001). The Food and Agriculture Organization estimates that about 25% of food crops in the world, including many staple foods are contaminated with mycotoxin-producing fungi (Rice and Ross 1994, Devegowda et al., 1998, Stepien et al., 2007). Patulin is a naturally produced by species of *Penicillium*, *Aspergillus* and *Byssochlamys* fungi (Baert et al., 2007). The major sources of contamination are apples and apple products, which are also the most important source of patulin in the human diet (Pitt and Hocking 1997, Reddy et al., 2010). Patulin exhibits a number of toxic effects in humans and other animals under varied conditions (Doi and Uetsuka,

2014; Frizzel et al., 2014), and is believed to be mutagenic, neurotoxic, genotoxic and immunotoxic to animals (Moake et al., 2005). Therefore, the patulin level in food has been limited in many countries around the world. In Morocco, several studies have been conducted to describe the occurrence of different mycotoxins and toxigenic fungi in foods (El adlouni et al., 2006; Roussos et al., 2006; Zinedine et al., 2007; Juan et al., 2008; Zinedine 2010). Little is known concerning the contamination by patulin compared to ochratoxin A or aflatoxins (Rharmitt et al., 2016). For cereals and transformation products consumed widely in Morocco, very few studies have been conducted to assess the degree of contamination by producing strains of patulin. A recent study in North-West of Morocco showed the presence of patulin-producing species of *Penicillium* isolated from durum wheat and barley field and also its transformation products (flour, semolina) (Mansouri et al., 2014). Among the strains producing patulin, a molecular identification and physiology study is conducted on a strain of *Penicillium* to evaluate the production potential of this mycotoxin. It has been reported that environmental conditions such as temperature, pH and water activity are important factors influencing patulin biosynthesis in *Penicillium*, *Aspergillus* and *Byssochlamys* (Northolt et al., 1978; Lopez dias and Flannigan 1997; McCallum et al., 2002; Garcia et al., 2011). Concerning the effects of C and N sources on patulin production, few works have been reported (Zong et al., 2015).

The objective of this study was to investigate the influence of carbon and nitrogen source on the physiology of *P. expansum* and patulin production. Several carbon and nitrogen sources were tested in order to optimize a chemically defined medium for this strain isolated from cereals and transformation products. We show here that although growth occurred on a large variety of substrates, the choice of nitrogen source and environmental conditions had a major impact on patulin production.

2. Materials and Methods

2.1. Microorganism and growth conditions

Stock culture of *P. expansum* (one of these trains isolated and identified by microscopic and molecular identification) was maintained on CZapek Agar (CZA) medium. The slants were stored at 4°C after growth on potato dextrose agar for 8 days at 28°C. The conidiospores were harvested with sterile solution (0.9% NaCl, 0.05 % Tween 80), washed twice with sterile buffer (20 mM KH_2PO_4, adjusted to pH 2.0 with HCl) and enumerated.

2.2. Molecular identification

To confirm the identity of the *P. expansum* strain, we have proceeded by a partial sequencing of the β -tubulin or Calmodulin genes. DNA extraction was performed using high-speed cell disruption method as described by Müller et al. (1998). Amplifications were carried out in a total volume of 25 µl containing 17.75 ml of diluted genomic DNA, 1µl of My Taq DNA Polymerase kit, 20 µL de My Taq Reaction Buffer and 4µl (10 mM) of each β-tubulin 01 (bt1a) and β-tubulin 2a (bt2a) primers (Glass and Donaldson, 1995). Amplification was performed on a Veriti thermal cycler (Applied Biosystems) using an initial denaturation of 95 °C for 3 min followed by 35 cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 10 s. A final extension step of 5 min at 72 °C was included. Amplicons were purified using the Exosap purification kit and sequenced with the ABI 3130 XL Sequencer (Applied Biosystems) using the same primers used for PCR. BLAST searches, against Gen Bank database, were carried out for taxonomic identification of our fungal strains using a threshold of ≥99% of similarity for *Penicillium* species delineation.

2.3. Culture media

2.3.1. Culture on complex medium

The following culture media were used in the present work: CZA medium: $NaNO_3$ 2 $g.L^{-1}$; $MgSO_4$ 0.5 $g.L^{-1}$; KCl 0.5 $g.l^{-1}$; $FeSO_4$ 0.01 $g.L^{-1}$; K_2HPO_4 1 $g.L^{-1}$; sucrose 30 $g.L^{-1}$; agar 15 $g.L^{-1}$; metallic solution 1 mL ($ZnSO_4$ 1 $g.L^{-1}$; $CuSO_4$ 0.5 $g.L^{-1}$; distilled water 100 mL) and 1000 mL distilled water. Liquid medium Czapek Yeast extract Agar (CYA): $NaNO_3$ 2 $g.L^{-1}$; $MgSO_4$ 0.5 $g.L^{-1}$; KCl 0.5 $g.L^{-1}$; $FeSO_4$ 0.01 $g.L^{-1}$; K_2HPO_4 1 $g.L^{-1}$; sucrose 30 $g.L^{-1}$; metallic solution 1 mL ($ZnSO_4$ 1$g.L^{-1}$; $CuSO_4$ 0.5 $g.L^{-1}$; distilled water 100 mL), yeast extract 5 $g.L^{-1}$; and 1000 mL distilled water. Liquid medium YES: yeast extract 20 g; sucrose 150 g; $MgSO_4$ 0.5 $g.L^{-1}$; and distilled water 1000 mL. The media were autoclaved at 121 °C for 20 min.

2.3.2. Culture on synthetic medium

The chemically defined fermentation medium developed in this study contained the following: Glucose or sucrose, at various concentrations (5, 10, 20 and 50 $g.L^{-1}$) as carbon source, sodium glutamate as nitrogen source 5 $g.L^{-1}$; K_2HPO_4 5 $g.L^{-1}$; KH_2PO_4 5 $g.L^{-1}$; $MgSO_4$ 0.5 $g.L^{-1}$; $FeSO_4$ 0.01 $g.l^{-}$

^{1};$CaCl_2$ 0.1 $g.L^{-1}$; $ZnSO_4$ 0.01 $g.L^{-1}$; $MnSO_4$ 0.03 $g.L^{-1}$. The media were autoclaved at 121 °C for 20 min. The pH of the medium was adjusted to 6.5 with HCl (2N) or KOH (2N).

2.3.3. Culture conditions

Fungi were grown in CZA for 10 days at 25 °C to obtain heavy sporulation. The liquid mediums were inoculated with the spores suspensions of each isolate prepared with a sterile distilled water. YES and synthetic medium were adjusted to pH 6.5. The reference medium CYA at pH 7.5 was adjusted to pH 4; 6.5 and 8 using HCl (2N) or KOH (2N). The CYA at pH 6.5 was incubated at the required temperature (20; 25; 30 and 35°C) for 10 days. Finally, the cultures media were incubated 10 days. The biomass was determined by gravimetric analysis after filtration of cell samples through pre-weighed nylon filters (45-mm diameter; pore size, 0.8 mm) and dried at 95°C to a constant weight.

2.3.4. Patulin extraction

The broth fermentation is obtained after 7 days of incubation the isolates in the static liquids mediums (CYA, YES, and Synthetic medium). The liquids cultures were filtered through a 0.45µm nitrocellulose membrane. The 15 mL of the filtrate was acidified with 100 µL concentrated HCl and mixed with 15 mL of chloroform. The organic phase was collected and concentrated to dryness in a rotary evaporator at 50 °C and the extract is taken up with 1 mL of methanol for the subsequent analysis.

2.3.5. Identification and quantification of Patulin

HPLC analysis of patulin was performed with a Waters 2695 pump, auto sampler and Waters 2998 photodiode-array detector (276 nm) with Spectra Manager software and Empower 3 Soft-ware data registration. The separation was achieved using a Waters Spherisorb ODS2 (5 µm, 250 × 4 mm) column. The system was used in isocratic mode with a mobile phase consisting of a mixture of water and acetonitrile (95:5, v/v) at a flow rate of 1 $mL.min^{-1}$. The injection volume of the standard and sample extract was 50 µl. All assays were performed at room temperature conditions. The quantification of patulin was performed by the measurement of the peak area at PAT retention time and the comparison with the relevant calibration curve (1; 0.75; 0.5; 0.25 and 0.1 $mg.mL^{-1}$ patulin standard). The pure Patulin was purchased from the Sigma Company.

3. Results

In this paper, the secondary metabolism of *Penicillium. sp* isolated from Moroccan cereals and its derived was studied. In order to increase the efficiency and accuracy of fungal identification of this fungus, a multiplex PCR assay was developed which is capable of identifying based on the banding pattern of amplified genes following agarose gel electrophoresis. The selected genes allow for specific amplification and specie identification. A multiplex PCR-based method and sequencing using a β-tubulin revealed that *Penicillium* species is identified as *P. expansum.* The latter was developed on both reference culture media CYA and YES. After 10 days of incubation at 25 °C, it has been shown that CYA medium is characterized by a basic pH (8.3) and represents a favorable substrate for specific patulin production (0.65 $\mu g.mg^{-1}$) (Table 1).

	CYA	**YES**
pH	8,3 ± 0.2	4.3 ± 0.2
Biomass $mg.ml^{-1}$	13± 1.5	16,5± 2.3
Specific patulin production ($\mu g.mg^{-1}$)	**0,65**± 0.1	**0,018**± 0.0

Table 1: Effect of culture media (CYA and YES) on specific patulin production during submerged fermentation of *P. expansum.*

3.1. Effect of physico-chemical parameters (pH and temperature)

In order to determine the effect of pH patulin production, mycelium of *P. expansum* was cultured in liquid media with pH values which ranged from 4 to 8 with a constant temperature (20°C). Maximum specific patulin production rate obtained by *P. expansum* was 1.76 $\mu g.mg^{-1}$ at pH 6.5. This specific patulin production was negatively affected by increasing pH (Figure 1a). In the experiments concerning the effects of temperature on patulin biosynthesis by *P. expansum*, the optimal temperature for the specific patulin production was around 20° C. Maximum patulin concentrations at 20°C were obtained as 4.2 $\mu g.mg^{-1}$. However, a further increase of temperature to 25℃ and 30℃ caused a decrease in patulin production (Figure 1b). These results proved that pH and temperature plays a role in patulin production but other extrinsic and intrinsic factors appear to interact.

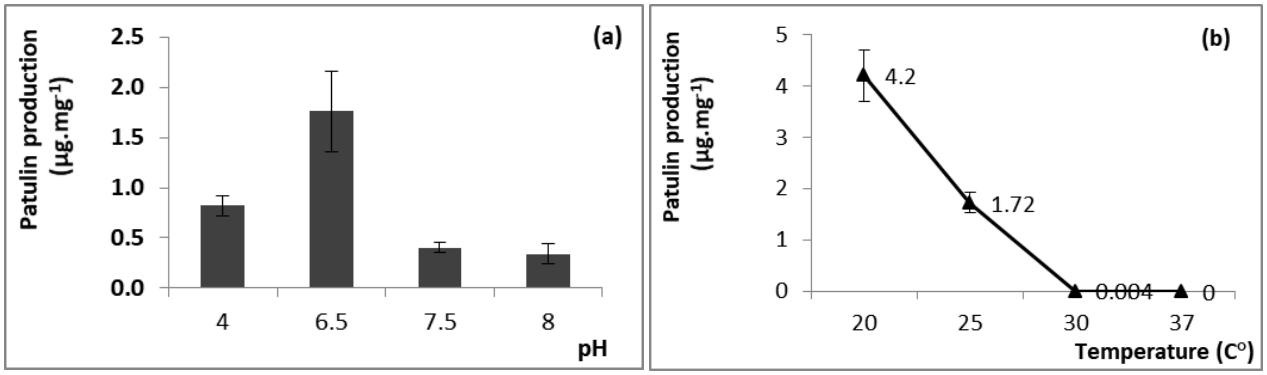

Figure 1: Effect of pH (a) and temperature (b) variation on specific patulin production during submerged fermentation of *P. expansum*. The data reported are from one typical experiment which was repeated at least twice with consistent results.

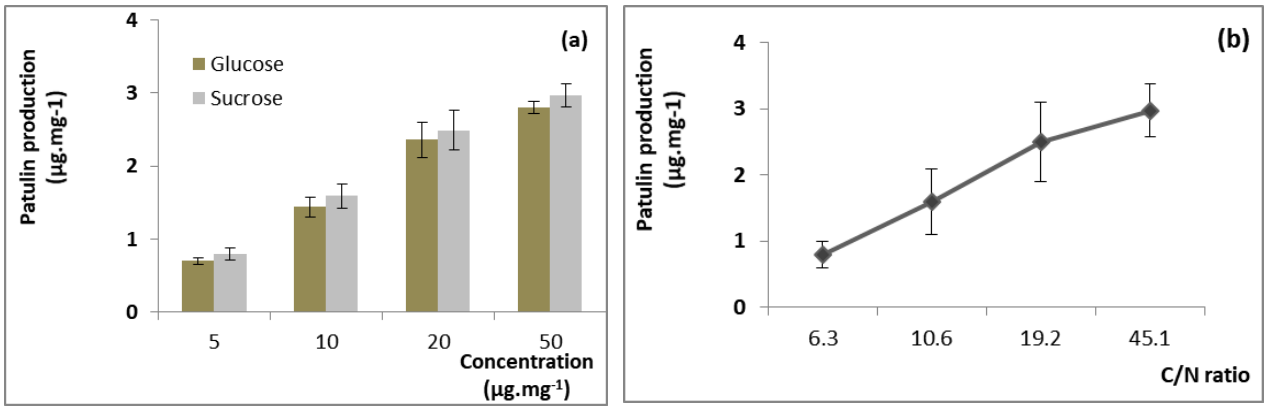

Figure 2: Profile effect of glucose and sucrose concentration (a) and C/N ration (b) on specific patulin production during submerged fermentation of *P. expansum*. The data reported are from one typical experiment which was repeated at least twice with consistent results.

3.2. Effect of nitrogen (N) and carbon (C) sources

The patulin production by *P. expansum* was studied under different C and N conditions. A total of seven different nitrogen sources including two ammonium, one nitrates, and fours organic N sources were tested in shake flask cultures using a defined medium with 5 $g.L^{-1}$ of glucose as the sole carbon source. In general, all the *P. expansum* strains showed better biomass formation and strong influence patulin production in media with nitrogen sources (Table 2). For inorganic nitrogen sources, ammonium nitrate was better for biomass and patulin production than ammonium chloride and sodium nitrate. They did not support patulin production (Table 2). For organic N effect (amino acids), L-alanine and L-glutamate were the best N sources for patulin production (0.66 $\mu g.mg^{-1}$) and (0.71 $\mu g.mg^{-1}$) respectively. L-Glutamate was better utilized by *P. expansum* for patulin production, compared to other three amino acids (0.71 $\mu g.mg^{-1}$) (Table 2). This effect is due to its degradation caused by H_2O_2 generated during assimilation of histidine. In general, organic N sources gave better yields of patulin than inorganic N sources. To investigate the C sources effect on specific patulin production, the *P. expansum* strain was cultured in media containing 5 $g.L^{-1}$ of L-glutamate used like nitrogen source and four different C sources (glucose, sucrose, acetate and ethanol), patulin production was compared after 10 days. The biomass growth varied when *P. expansum* was cultured with different C sources (Table 3), glucose, sucrose and ethanol was better for biomass growth 1.6, 1.8, and 1.5 $mg.mL^{-1}$ respectively. Patulin production was also strongly influenced by C sources. *P. expansum* showed the highest specific patulin production (0.77 $\mu g.mg^{-1}$) with sucrose as sole sources of C followed by glucose and ethanol

Nitrogen sources	Biomass $mg.ml^{-1}$	Specific patulin production ($\mu g.mg^{-1}$)
NH_4NO_3	2,2 ± 1	0,14 ± 0.0
NH_4Cl	2 ± 0.5	N.D
$NaNO_3$	1,4 ± 0.6	N.D
L-Leucine	1,9 ± 0.8	0,04 ± 0.0
L-Alanine	1,5 ± 0.8	0,66 ± 0.1
L-Histidine	1,8 ± 1.1	0,1 ± 0.0
L-Glutamate	1,7 ± 0.8	0,71 ± 0.1

Table 2: Effect of nitrogen sources on specific patulin production during submerged fermentation of *P. expansum*.
N.D: Not detected

Carbon sources	Biomass $mg.ml^{-1}$	Specific patulin production ($\mu g.mg^{-1}$)
Glucose	1,6 ± 0.5	0,68 ± 0.2
Sucrose	1,8 ± 0.7	0,77 ± 0.1
Sodium acetate	0,6 ± 0.2	0,03 ±0.0
Ethanol	1,5 ± 0.9	0,55 ± 0.1

Table 3: Effect of carbon sources on specific patulin production during submerged fermentation of *P. expansum*.

(Table 3). On the contrary, *P. expansum* gives a very low specific patulin production in presence of acetate (0.03 $\mu g.mg^{-1}$).

3.3. Effect of carbon concentration and carbon/ nitrogen ratio

The liquid fermentation of *P. expansum* was made in shaking flask experiments with glucose and sucrose concentrations which range between 5 and 50 $g.L^{-1}$ and the concentration of N source (L-glutamate) which was fixed at 5 $g.L^{-1}$. Figure 2a shows variation of glucose and sucrose concentration and specific patulin production. The patulin production increased considerably with the increase of carbon sources concentration (Figure 2a). Maximum specific patulin production was observed for 50 $g.L^{-1}$ concentration of sucrose (2.97 $\mu g.mg^{-1}$) and glucose (2.80 $\mu g.mg^{-1}$). To elucidate the Carbon/Nitrogen (C/N) ratio effect on specific patulin production by *P. expansum*, four different sucrose concentrations at constant nitrogen concentrations (5 $g.L^{-1}$) were tested in shaking flask experiments: 5, 10, 20 and 50 $g.L^{-1}$ which corresponds to a C/N ratio of 6.3, 10.6, 19.2 and 45.1 respectively. Figure 2b shows that patulin concentration at the respective C/N ratio influences strongly the patulin biosynthesis in *P. expansum* (Figure 2b).

The specific patulin production is positively correlated to C/N ratio.It is increased with the increase of C/N ratio. Our results go hand in hand with those which showed that C/N ratio has a positive impact on citrinin production by *M. ruber* (Hajjaj et al., 2015). Indeed, Casas Lopéz et al. (2003) studied the influence of different C/N ratio on lovastatin production. They showed that the presence of excess C under N limitation greatly enhanced the rate of production of lovastatin. In another report, it was found that an excess of C sources has a positive impact on alternariol production but does not affect growth rate (Brzonkalik et al., 2012).

4. Discussion

Cereals and derived products contaminated by patulin or other mycotoxins are harmful to human and animal health. In this study, the effects of culture medium, pH, temperature, N sources, C sources and C/N ratio factors were elucidated, and it was shown that patulin production was highly affected by these factors. It has been shown that culture medium was the important factor for mycotoxin biosynthesis. CYA medium is the best environment for the mycotoxin production and was subsequently chosen to study the impact of physico-chemical and nutritional factors on the patulin biosynthesis (Abramson et al., 1996). In general, the regulation of most of fungal toxins is very complex and one special regulator does not exist (Brzonkalik et al., 2012). As shown by many studies before, pH exerts a great influence on the production of different mycotoxins and most of them are increased at acidic pH values (Keller et al., 1997). The current study shows that patulin production was negatively affected by increasing pH (Zong et al., 2015). Our results show that pH at 6.5 was the most favorable for patulin production. The latter is not similar to the results of Keller et al. (1997) and Merhej et al. (2011) which reported that acidic pH values above 3-5 led to an optimal mycotoxin production. Concerning the effects of temperature, it has been described that the optimum specific patulin production is located near 17°C (Paster et al., 1995). Similar results have been reported for other fungal species like *A. niger* and *A. carbonarius* which produced ochratoxin A at 15 °C on maize seed (Alborch et al., 2011). The secondary metabolites production may be influenced by changing physical, chemical and biological factors, and by interactions involving these factors. Chief among which are carbon and nitrogen sources (Bourass et al., 2016). The nature and concentration of the C and N sources are primary factors that interact to affect mycotoxin biosynthesis (Icgen et al., 2002). The *P. expansum* cultured in the study was able to grow on synthetic media supplemented with different N sources (including organic and mineral sources). This indicates that the isolated *P. expansum* is adapted to different N sources. However, patulin production varies with N sources, the presence of amino acid L -alanine and L-glutamate in synthetic medium resulted in increased production of patulin relative to its production in the presence of mineral nitrogen sources. These results are consistent with those of Zong et al. (2015), who reported that organic nitrogen sources were more favorable for the produc-

tion of some secondary metabolites, such as L-alanine compared to inorganic N sources. Ammonium or nitrate alone inhibits the mycotoxin production from polyketides types (Brzonkalik et al., 2012). The effect on patulin production by glutamate was also observed by Zong *et al*. (2015). A similar effect of alanine was observed by Bouras et al. (2016) who showed that L-alanine was the most favorable N source for mycotoxins production by *Pyrenophoratritici-repentis*. Conversely, a low specific patulin production was detected in presence of L-histidine (0.19 $\mu g.mg^{-1}$), and only traces of patulin in the fermentation medium (<0.08 $\mu g.mg^{-1}$) was detected in presence of L-leucine. Similar results have been reported by *M. ruber* which produces citrinin, it is totally absent when histidine was used as the sole N sources (Hajjaj et al., 2012, Silpha et al., 2015). For the different C sources effect, patulin production was variable with different C sources. Glucose and sucrose were the most suitable C sources for maximum patulin production compared with other carbon sources. Our results are in agreement with those of Zong et al. (2015) who reported that sucrose was the most favorable for patulin production. Also, the patulin production is increased with the increase of carbon sources concentration. The same results were reported by Hashem et al., (2015) who showed that glucose, sucrose, mannose, maltose and fructose promote patulin and ochratoxin A production. Although the influence of different nutritional and environmental factors were studied extensively on biosynthesis of patulin by different species of *Penicillium* species, the impact of other factors such as C/N ratio was neglected so far. It was shown that C/N ratio influences strongly the patulin biosynthesis in *P. expansum*. The C/N ratio is an important factor that influences the fungal growth, sporulation and the secondary metabolites production (Gao et al., 2007). Brzonkalik et al. (2012) showed that mycotoxin production by *Alternaria alternata* was depending on different C/N ratio in submerged cultivation. This study demonstrated that the different nutritional and physico-chemical parameters tested in the defined media strongly influence the specific patulin production in *P. expansum*.

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Conflict of Interest

No conflict of interest.

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