Effect of Solid Substrate, Liquid Supplement, and Harvest Time on 6-*n*-Pentyl-2*H*-pyran-2-one (6PAP) Production by *Trichoderma* spp.

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Selected strains of *Trichoderma* spp. were grown on 10 different solid media substrates and analyzed for the production of 6-*n*-pentyl-2*H*-pyran-2-one (6PAP). Using the highest yielding medium from this study, ground corn plus a liquid supplement, 15 *Trichoderma* isolates were screened, and this led to the identification of two high yielding strains. By selection of solid media, liquid supplement, and harvest time, the yield of 6PAP was improved from ca. 200 up to about 1000 mg/kg. These results will provide an indication for future production studies leading to the development of a bulk-scale fermentation process.

Keywords: *Trichoderma; 6-pentyl-α-pyrone; 6PAP; metabolite; production; culture*

INTRODUCTION

Trichoderma species produce a number of antibiotics, including 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) (Figure 1), a polyketide possessing a characteristic coconut-like aroma (Collins and Halim, 1972; Cutler et al., 1986). Due to its potent antifungal activity, this metabolite has attracted considerable interest over recent years as a potential biocontrol agent (Ghisalberti and Sivasithamparam, 1991; Cutler and Hill, 1994).

6PAP has shown promising in vitro and in vivo control of several major phytopathogenic fungi which affect New Zealand grown crops including *Armillaria*, *Botrytis*, *Phytophthora*, and sapstain fungi (Cutler and Hill, 1994). It also has the added advantage of low mammalian toxicity and approval for food use by the USFDA and is currently being tested as a natural fungicide.

The ability of *Trichoderma* isolates to produce 6PAP has been found to differ between isolates of different species as well as between isolates of the same species (Dennis and Webster, 1971a,b). For a particular isolate, culture conditions have also been shown to affect the quantity of the metabolite produced. For example, using liquid cultures of *T. viride*, Zeppa et al. (1990) found that both the culture substrate and the method of culturing, i.e., surface or submerged, influenced the quantity of the pyrone derivatives produced. This has major implications for the role of *Trichoderma* isolates as biocontrol agents.

6PAP can be prepared both synthetically in the laboratory and naturally via fermentation of *Trichoderma*, but it is a high-value compound and supplies are extremely limited. The aim of the present work has been to identify high 6PAP yielding strains of *Trichoderma* and to optimize culture conditions for the production of the metabolite leading to the development of an economic bulk fermentation process.

MATERIALS AND METHODS

Apparatus. Culture samples were incubated in a Sanyo MIR 252 incubator fitted with fluorescent lights operating continuously. Gas chromatography-flame ionization detection (GC-FID) analysis for 6PAP was carried out on a HRGC 5300 Mega Series Carlo Erba (Milan, Italy) fitted with a Hewlett-Packard (Avondale, PA) HP-5 capillary column, 25 m × 0.20 mm i.d. (0.33 μm film thickness). Injector and detector temperatures were 250 °C. The GC oven temperature was held at 180 °C for 6 min, then programmed at 30 deg/min to 230 °C, and then held for a further 10 min. The retention times of 6PAP and the internal standard, hexadecane, were 3.7 and 5.1 min, respectively.

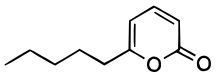

Figure 1. 6-*n*-Pentyl-2*H*-pyran-2-one (6PAP), a secondary metabolite produced by *Trichoderma* spp.

Reagents and Materials. All solvents were of analytical grade or better and were supplied by BDH Ltd (NZ). Potato dextrose agar (PDA) was obtained from Gibco BRL Life Technologies (Paisley, Scotland), and bacteriological agar was supplied by Scientific Supplies Ltd (NZ). Malt was obtained from NZ Food Industries Ltd (NZ) and yeast extract and caseine hydrolysate from DIFCO Laboratories (Detroit MI). Luria broth was supplied by Life Technologies (NZ). KH_2PO_4, $MgSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, KCl, $NaNO_3$, glucose, and sucrose were purchased from BDH Ltd (NZ). Corn steep liquor (CSL) was obtained from a commercial wet milling plant (NZ Starch Products Ltd), while brewers sugar and corn syrup were both of food grade.

Trichoderma spore suspensions were prepared in sterile water using the surface growth from freshly grown plates. Spore concentrations were determined using a Hawksley haemocytometer (depth 0.1 mm 1/400 mm^2). Malt 3.5 agar (malt agar, modified by adjusting the pH to 3.5 by the addition of lactic acid) (Atlas, 1993) was used for all agar culture samples unless stated.

***Trichoderma* Isolates.** A total of 15 *Trichoderma* isolates (labeled A−O) identified as *T. hamatum* Rifai (eight), *T. harzianum* Rifai (five), and *T. koningii* Rifai (two) (supplied by R. A. Hill, HortResearch, Hamilton, New Zealand) were used during the course of this study.

General Culture Conditions. For solid culture samples, the *Trichoderma* isolates were grown in 200 mL conical flasks on sterilized solid media (10 g), treated with (unless otherwise stated) liquid supplement (5 mL) and water (3 mL), and inoculated using 1 mL of prepared spore suspensions with measured concentrations in the range 1×10^8 to 1×10^9 spores/mL. Flasks were incubated at 20 °C. For agar culture samples, the *Trichoderma* isolates were grown on malt 3.5 plates (9 cm diameter) inoculated with prepared spore suspensions with measured concentrations in the range 1×10^8 to 1×10^9 spores/mL (100 μL). Agar plates were incubated at 20 °C. For liquid culture samples, *Trichoderma* isolates were grown in 200 mL conical flasks containing liquid culture media

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Table 1. Composition of Media Used to Test the Effect of Substrate on 6PAP Production

substrate media	composition
agar 1	PDA (10 g) and malt (100 g) made up to 500 mL with water
agar 1 + supplement	yeast extract (10 g) made up to 100 mL with Agar 1
agar 2	bacteriological agar (10 g) and malt (15 g) made up to 500 mL with water
agar 2 + supplement	yeast extract (10 g) made up to 100 mL with Agar 2
broth	yeast extract (50 g) and malt (100 g) made up to 500 mL with water
whole wheat	whole wheat (10 g) and water (6 mL)
ground wheat + supplement	ground wheat (10 g), yeast extract solution (10%, 5 mL), and water (2 mL)
ground corn + supplement	ground corn (10 g), yeast extract solution (10%, 5 mL), and water (3 mL)
bark	sieved *Pinus radiata* bark (particle size <2 mm, 10 g) and water (10 mL)
bark + supplement	sieved *Pinus radiata* bark (particle size <2 mm, 10 g), yeast extract solution (10%, 5 mL), and water (5 mL)

(10 mL) and inoculated with prepared spore suspensions with measured concentrations in the range 1×10^8 to 1×10^9 spores/mL (1 mL). Flasks were gently agitated on an orbital shaker at ca. 20 °C.

Effect of Substrate on 6PAP Production. Three *Trichoderma* isolates (A, I, and O) were grown on 10 different substrate combinations (seven different substrates and five with added 10% yeast extract supplement) (see Table 1). The cultures were extracted on day 19 and analyzed for production of 6PAP. Two further isolates, B and G, were grown on four of the substrate combinations (broth; ground wheat + supplement; ground corn + supplement; bark + supplement) and extracted after 17 days. All culture samples were grown in duplicate.

Comparison of Isolates. Fifteen isolates (A−O) were grown on ground corn plus 10% yeast extract supplement and on malt 3.5 agar plates. The culture samples were extracted after 19 days and analyzed for the presence of 6PAP production. Culture samples were prepared in duplicate.

Production of 6PAP with Time. To determine the optimum time window in which to harvest 6PAP from the culture samples, two isolates, A and B, were grown on ground corn + 10% yeast extract and on malt 3.5 agar plates. Twenty replicates of each isolate/media combination were prepared and duplicate samples analyzed every 2−3 days.

Effect of Liquid Supplement on 6PAP Production. Two isolates, A and B, were grown on substrates consisting of ground corn treated with one of 10 different liquid supplements (see Table 2). The B culture samples were extracted on day 11 and the A culture samples after 17 days. Isolate A was also cultured on 10 mL aliquots of solutions 7 and 8 and luria broth gently agitated for 17 days on an orbital shaker before extraction.

Extraction of Culture Samples. Solid culture samples were extracted by adding methanol−water (85:15, 50 mL) to the substrate representing 10 g of solid material. Agar and liquid culture samples were extracted by placing the total sample (ca. 15 g of agar + mycelium or 10 mL of liquid media + mycelium) into methanol−water (85:15, 50 mL). Solid, agar, and liquid samples were blended with a Polytron homogenizer for ca. 1−2 min so that no visible lumps remained, then allowed to stand for ca. 4 h. Each sample was filtered through a Whatman Glass Fibre Filter (GF/A; 9 cm diameter). An aliquot of the extract solution (4 mL) was added to cyclohexane (2 mL) and buffered 10% saline solution (pH 6.5, 10 mL), then mixed and allowed to partition overnight. The cyclohexane layer was separated and dried by passing it through anhydrous Na_2SO_4 (ca. 1.5 g). An aliquot (1 mL) of the dried cyclohexane fraction was spiked with a 22 ppm hexadecane internal standard solution (100 μL) and analyzed for 6PAP by GC-FID.

The partition step of the analytical method was used to provide a cleanup prior to GC-FID analysis (especially useful for the solid substrates) and to transfer the analyte into a GC-amenable solvent. The overall recovery of 6PAP was determined for the most utilized media (ground corn) and found to be consistently in the range 67−74%. Results presented in this paper are as recorded, and for estimates of absolute yield values, a scaling factor of 1.4 should be applied.

Table 2. Composition of Liquids Used to Test the Effect of Supplements on 6PAP Production in a Corn Matrix

liquid supplement	composition
brewers sugar	brewers sugar (4 mL) and water (4 mL)
corn syrup	corn syrup (4 mL) and water (4 mL)
CSL#306	high mycotoxin corn steep liquor (CSL)#306[a] (4 mL) and water (4 mL)
CSL#215	high mycotoxin corn steep liquor (CSL)#215[b] (4 mL) and water (4 mL)
CSL#BS	low mycotoxin corn steep liquor (CSL)#BS[c] (4 mL) and water (4 mL)
luria broth	bactotryptone, yeast, and NaCl (25 g/L, 4 mL) and water (4 mL)
yeast extract solution	yeast extract solution (10%, 5 mL) and water (3 mL)
solution 7	(Zeppa et al., 1990) (4 mL) containing yeast extract (1 g/L), caseine hydrolysate (2 g/L), KH_2PO_4 (1.5 g/L), $MgSO_4 \cdot H_2O$ (1 g/L), and glucose (15 g/L) and water (4 mL)
solution 8	(Moss et al., 1975) (4 mL) containing $NaNO_3$ (2 g/L), KCl (0.5 g/L), KH_2PO_4 (1 g/L), $MgSO_4.7H_2O$ (0.5 g/L), $FeSO_4 \cdot 7H_2O$ (0.1 g/L), and sucrose (30 g/L) and water (4 mL)
water	water (8 mL)

[a] Containing the *Fusarium* mycotoxins nivalenol (NIV, 4.9 mg/kg) and deoxynivalenol (DON, 4.9 mg/kg). [b] Containing NIV (8.4 mg/kg) and DON (8.9 mg/kg). [c] Containing DON (0.53 mg/kg).

Table 3. Comparison of 6PAP Production (mg/kg or mg/L) on 10 Different Media, and Including the Effect of Added Yeast Extract Supplement[a]

substrate media (10 g or 10 mL)	A	I	O	B	G
agar 1[b]	58	92	nd		
	63	99	nd		
agar 1 + supplement	nd	nd	nd		
	32	nd	nd		
agar 2[c]	nd	nd	nd		
	32	nd	nd		
agar 2 + supplement	nd	23	nd		
	nd	17	nd		
broth[d]	8	57	nd	83	nd
	12	nd	nd	61	nd
whole wheat	105	60	13		
	112	88	16		
ground wheat + supplement	203	136	97	351	nd
	195	88	47	413	nd
ground corn + supplement	923	583	17	881	nd
	873	542	213	681	nd
bark[e]	nd	nd	nd		
	nd	nd	nd		
bark + supplement	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd

[a] nd: nondetected (<3 mg/kg 6PAP). [b] Agar 1: PDA and malt. [c] Agar 2: bacteriological agar + malt. [d] Broth: yeast extract + malt. [e] Bark: sieved *Pinus radiata* bark (particle size <2 mm).

RESULTS

Effect of Substrate on 6PAP Production. The results presented in Table 3 show that 6PAP was produced by isolates A, I, O, and B, with the highest production by isolate A, followed by isolates B and I. Ground corn supplemented with a 10% yeast extract solution gave the highest levels of 6PAP production for all the isolates tested.

Comparison of Isolates. Figure 2 shows comparative 6PAP yield data for 15 *Trichoderma* isolates cultured on ground corn and agar media. Only six isolates produced 6PAP on agar (concentration range 17−202 mg/kg), while 14 produced on corn, with yields

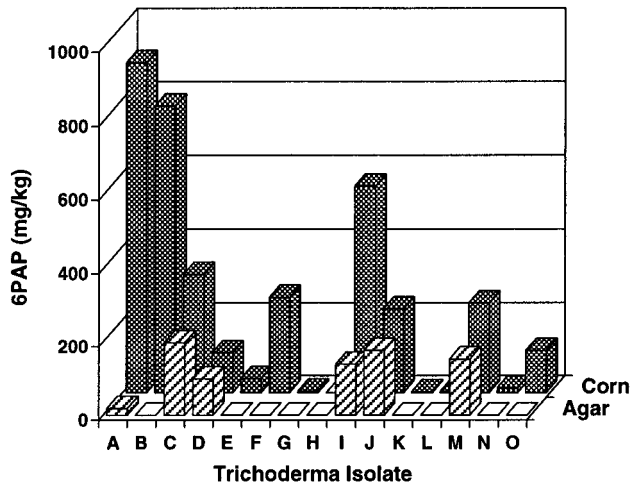


Figure 2. Comparison of 6PAP production (mg/kg) on ground corn + 10% yeast extract supplement and on malt 3.5 agar plates for a range of *Trichoderma* isolates.

ranging from 5 up to 923 mg/kg. Isolates A and B were the highest yielding strains, giving levels of 923 and 681 mg/kg, respectively, on corn. Isolate H did not produce 6PAP on either media.

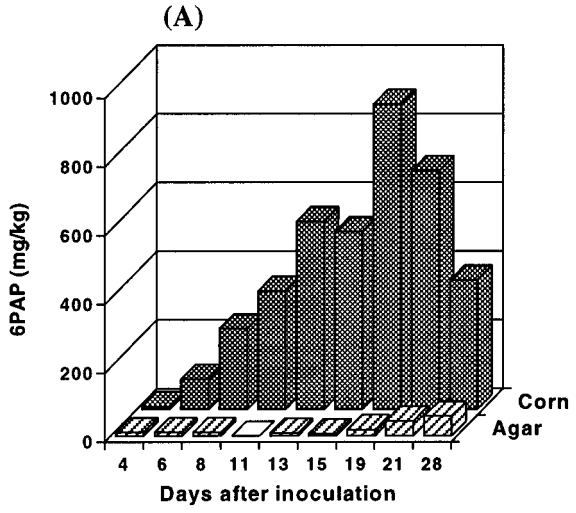
Production of 6PAP with Time. The 6PAP time harvest results are shown in Figure 3, parts A and B, for isolates A and B, respectively, cultured on both corn and agar media. For both isolates, 6PAP was detected within 4 days from the date of inoculation, albeit at low levels for A. The measured concentration of 6PAP was maximum at about 19 days for A when grown on corn and at about day 11−15 for B. For both isolates, longer incubation times caused a drop in the measured concentrations of 6PAP. On malt 3.5 agar plates, the levels of 6PAP detected remained very low until day 21 for A, while for B, the levels peaked at days 11−15 before dropping off rapidly to below the level of detection.

Effect of Liquid Supplement. Results showing comparative effects of liquid supplements on 6PAP production are presented in Figure 4. Harvest times for the two isolates were chosen in accord with the time windows suggested from the time harvest study (above).

Both isolates produced only low levels of 6PAP on ground corn supplemented with either brewers sugar, corn syrup, or the three corn steep liquor (CSL) examples. For these supplements, the 6PAP concentrations were significantly less than that recorded when using only water as the liquid supplement, which suggests that 6PAP production was being retarded. Of the three CSL samples tested, the highest production of 6PAP was observed for CSL#BS which had the lowest mycotoxin contamination.

Of the remaining supplements, only solutions 7 and 8 enhanced 6PAP production for both isolates above that obtained for water. Luria broth and 10% yeast extract solution also improved 6PAP yields for isolate B but had little effect for A.

Liquid culture samples of solutions 7 and 8 and luria broth inoculated with isolate A and gently agitated for 17 days on an orbital shaker gave no significant levels of 6PAP.



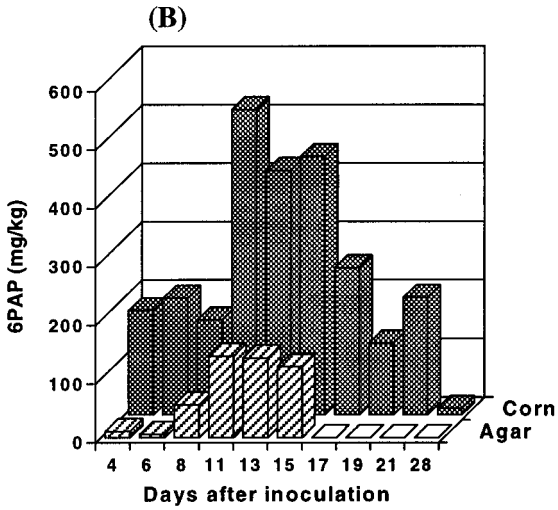


Figure 3. Production of 6PAP with time by *Trichoderma* isolates grown on ground corn with added 10% yeast extract solution and on malt 3.5 agar plates for (A) isolate A and (B) isolate B.

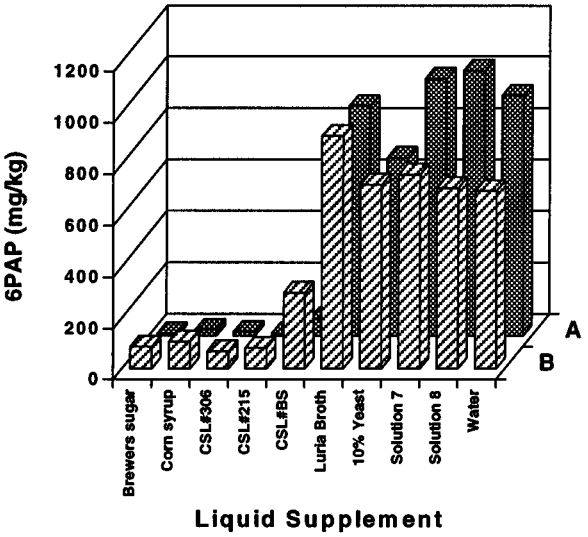


Figure 4. Effect of liquid supplement added to ground corn on 6PAP production by *Trichoderma* isolates A and B.

DISCUSSION

Most references to 6PAP production in the literature have concentrated on liquid fermentation techniques as opposed to solid fermentation processes. For example, previous work using liquid surface cultures and agitated submerged cultures of *Trichoderma* to study production of the coconut-like aroma, showed that the sources of carbon and nitrogen and the culture method can influence the quantity of 6PAP produced (Yong et al., 1985; Yong and Lim, 1986). The results were described using

GC peak area rather than absolute yield data, so no direct comparison is possible. Zeppa et al. (1990) found that glucose and ammonium sulfate stimulated the biosynthesis of 6PAP and underlined that the presence of ammonium nitrogen increases the production of 6PAP independently from the source of carbon present in the substrate for surface and submerged cultures. In accord with Yong and Lim, these workers found that submerged cultures produced less 6PAP than surface cultures, reporting yields of 17−27 and 14−78 mg/L, respectively. Moss et al. (1975) quoted yields of 10−30 mg/L for ether extracts from a *T. viride* isolate cultured on modified Czapek Dox medium identical in formulation to solution 8, while Collins and Halim (1972) report crude 6PAP yields as high as 180 mg/L for a *T. viride* isolate grown in a potato dextrose liquid medium.

A closer examination of solid-state fermentation processes in recent years in several research centers throughout the world has led to the realization of numerous economical and practical advantages of solid-state fermentation (Lonsane et al., 1992). These include superior productivity, low capital investment, reduced energy requirement, low wastewater output, improved product recovery, and elimination of foam products (Lonsane et al., 1985). The results of our studies indicate that solid-state fermentation of selected culture media by *Trichoderma* is capable of supplying 6PAP in good yield.

The production of 6PAP by *Trichoderma* was found to be superior when grown on solid media than on those liquid systems tested, and the concentrations obtained were also significantly higher than those reported in the literature for liquid media. Nevertheless, optimum yields were only achieved by correct choice of solid substrate. In this study ground corn was found to be the preferred medium. Production could be further enhanced by addition of a liquid supplement, although the results also showed that addition of either concentrated sugar substrate or corn steep liquor substantially reduced the yield of 6PAP. The time from inoculation to harvest was also found to be important for maximum yield. This was dependent on both the particular *Trichoderma* isolate used and the medium. The substantial drop in 6PAP concentration which was observed to occur after the optimum time suggests further metabolism of 6PAP by the *Trichoderma*. Since the GC-FID chromatograms did not show enhancement of minor peaks or the appearance of extra peaks concomitant with the reduction in 6PAP levels, it suggests that polar metabolites unlikely to be extracted by our analytical method were being produced. Oxygenated derivatives such as 3-(2-pyron-6-yl)propanoic acid and 5-(2-pyron-6-yl)pentanol have been shown to be produced during metabolism of 6PAP by the pathogenic fungi *Botrytis* (Poole and Whitaker, 1996). Further studies aimed at isolating and identifying the metabolites which may be produced as a result of 6PAP metabolism by the *Trichoderma* are planned.

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