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Bioreactor for continuous synthesis of compactin by *Penicillium cyclopium*

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Compactin was synthesized by *Penicillium cyclopium* in submerged as well as in bioreactor systems and assayed spectrophotometrically with a detection limit of 0.5 μg ml^{-1} solvent. Synthesis in submerged culture was affected by aeration, glucose level, pH, and type and molarity of buffer. Citrate or succinate (pH 4.0, 0.10 M) in malt glucose peptone broth (MGPB) stimulated cell specialization, sporulation, enhanced compactin permeation from mycelia and its production (60.05 μg ml^{-1} after 12 days). Fungal spores, immobilized onto-into loofah sponge, in a bioreactor, using MGPB-citrate as feed stock, resulted in productivity of 23.04 mg compactin (L^{-1} h^{-1}) during 50 days operation at 0.45 h^{-1} dilution rate. Compactin synthesis in the bioreactor was also affected by culture age, substrate, incubation and dilution rates. Scanning electron micrographs of the loofah sponge, prior to, during and post-spores immobilization showed that loofah channels served well for fungal support in the bioreactor.

Keywords: compactin; *Penicllium cyclopium*; antihypercholesteremic agent; bioreactor for compactin; continuous synthesis

Introduction

Fungi produce secondary metabolities during growth in response to environmental stress, and these compounds are synthesized from precursors derived from primary metabolism [2,5,60,62] and can be of value as growth regulator or antifungal, antibacterial and hypocholesterolemic agents [1,8,10,12,16–18,25,34,40,49,61]. Compactin (a lovastatin analogue), isolated from several fungi [4,8,12,17], functions as a specific competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase [7]. This compound and others, such as simvastatin and pravastatin [25] lowered serum cholesterol [21,22,29,51,52]. Compactin is an antihypercholesterolemic agent which affects DNA replication [15,36] and enhances adhesion of tumor cells [45]. Structural modifications are under investigation, to reduce toxicity [14,20,24,30,31,44,49,51–54,57]. Cyclopenol and cyclopenin are also produced by *P. cyclopium* [8,23,27,35,50]. This investigation was conducted to optimize compactin synthesis produced by *P. cyclopium* NRRL 6233, during submerged growth and continuously in a bioreactor using spore cells immobilized onto-into loofah sponge as carrier support. Factors affecting compactin synthesis, under both systems, were examined and scanning electron micrographs were also made on the loofah sponge both before and after cell immobilization.

Materials and methods

Materials, reagents and media

Oxoid potato dextrose agar (Basingstoke, Hants, UK); Nabisco shredded wheat (East Hanover, NJ, USA); loofah sponge (local market); silica gel 60 F-254 plates (Merck, Darmstadt, Germany); compactin from Dr Chu (Col Pharm, Athens, GA, USA); cyclopenol, cyclopenin and viridicatin from Dr Cutler (USDA, Athens, GA, USA). All chemicals were reagent grade and the highest purity available. Anisaldehyde reagent consisted of: methanol, 85 ml; concentrated sulfuric acid, 5 ml; and *p*-anisaldehyde, 0.5 ml [59]; Părducz solution had osmium tetraoxide (4%, w/v), and saturated mercuric chloride solution using 3:3:1, v/v/v, respectively; anthrone reagent contained 0.2 g anthrone in sulfuric acid and water (95:5, v/v). Semisolid medium [8,12] contained (g L^{-1} distilled water, DW): mycological broth (pH 4.8), 50; yeast extract, 20; and sucrose, 200. Two hundred milliliters of this broth were added to 100 g shredded wheat in Fernbach flasks (2.8 L) and sterilized (45 min, 121°C). Three types of liquid media were also used: malt glucose peptone broth (MGPB) [17], glucose basal salt broth (GBSB) [50]; and sucrose yeast extract mycological broth (SYEMB) [8] and contained (g L^{-1} DW): MGPB—malt extract, 30; glucose, 20; and peptone, 1. GBSB—glucose, 100; Na-citrate 38.2; NH_4Cl, 3.8; $CaCl_2 \cdot 2\,H_2O$, 2.1; $MgSO_4 \cdot 7\,H_2O$, 0.15; KH_2PO_4, 0.13; KCl, 0.05; $FeSO_4 \cdot 7\,H_2O$, 0.05; $ZnSO_4 \cdot 7\,H_2O$, 0.02. SYEMB—sucrose, 200; mycological broth, 50; and yeast extract, 20. All media, unless otherwise stated, were adjusted to pH 5.5 and 0.0125% sterile poly-propylene gylcol (MW 2000) was added.

Culture and conidiospores

An aberrant *P. cyclopium* strain (NRRL 6233) from Dr Cutler was kept on potato dextrose agar slants (Difco, Detroit, MI, USA) and was used to inoculate several Difco mycological agar slants (200 ml each, pH 4.8). Slants were incubated for 7 days at 25°C. Conidiospores were harvested, combined, microscopically counted (3.0×10^9 spores ml^{-1}), stored at 4°C and used. This aberrant strain deviated from the normal strain in the ability to synthesize secondary metabolites in fermentation.

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Submerged culture and factors involved

Fermentation vessels (1.8 L each) containing the desired medium, each equipped with an air sparger supplying 1.2 L sterile air/1.2 L broth min^{-1}, were inoculated and incubated at 25°C. At intervals, cell mass was monitored, dried, weighed, and the filtrate was assayed for reducing sugars, pH, total carbohydrate, compactin and other metabolites. Factors affecting submerged culture which were examined were: air flow into sparger; glucose level; pH of buffered and unbuffered MGPB; buffer type, and molarity.

Isolation of metabolites

Fernbach flasks (total 54), containing mycological broth with shredded wheat, were inoculated, kept for 14 days at 25°C, and culture media in all flasks were homogenized with acetone, the homogenate was filtered, acetone was evaporated, the pH adjusted to 4.0 and the filtrate was again extracted twice with ethyl acetate. The combined extracts were dried, concentrated (40°C), chromatographically assayed (TLC) using silica gel 60 F-254 plates (5 × 10 cm) and methylene-chloride-acetone mixture (85:15, v/v ratio) as developing solvent and metabolite standards were used for comparison. Plates were viewed under UV-254 nm or after 2 min heating following spraying with anisaldehyde reagent. The concentrated extract was also fractionated on a silica gel 60 prep-column (9.0 × 23.0 cm) and consecutively eluted using 1 L each of: benzene, tert-butyl methyl ether, ethyl acetate, acetone and acetonitrile. Aliquots of each solvent fraction were concentrated and assayed (TLC). The tert-butyl methyl ether soluble fraction was concentrated (40°C) and subjected to separation and to the purification procedure to isolate compactin and other metabolites (Figure 1). The isolated compactin was subjected to proton nuclear magnetic resonance analyses (^{1}HNMR), compared to standard (98.0% pure) compactin and percent purity determined. The spectra were recorded (20°C) on a Jeol FX90Q Fourier Transform 90 MHZ ^{1}HNMR spectrometer (Tokyo) using specified acquisition conditions. One compound (R_f 0.09) was subjected to low resolution electron impact mass spectral analysis using a Hewlett packard 5985 spectrometer (Palo Alto, CA, USA), introduced by a direct insertion probe following specified conditions and the spectrum was compared with reference library standards.

Assay of metabolites

Solubilities and UV spectra of isolated metabolites, in different solvents, were examined using a Hewlett Packard 8451A diode array spectrophometer and the concentrations determined as follows: 60 ml filtered wort was adjusted to pH 4.0, extracted twice with ethyl acetate, the ethyl acetate

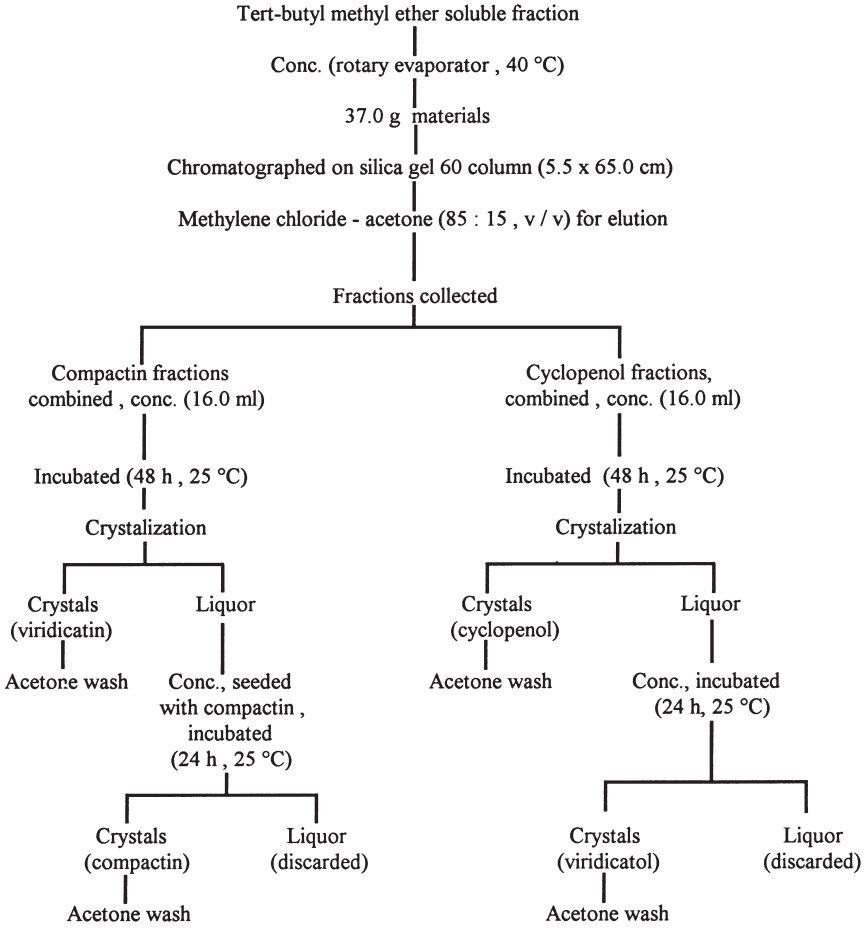

Figure 1 Schematic diagram for isolation of fungal metabolites (cyclopenol, viridicatin, compactin, and viridicatol) from the tert-butyl methyl ether soluble fraction.

phases were combined, dried, again extracted with acetonitrile, centrifuged and the supernatant phase was scanned from 200 to 330 nm. When compactin and cyclopenol were present, the absorbencies at 238 and 286 nm were used. If compactin and cyclopenin were present in the semisolid shredded wheat media (SWM), they were separated using two-dimensional high performance thin layer chromatography (HPTLC [11]) on silica gel 60 F-254 plates (10×10 cm). Benzene-acetone-acetic acid. (50:50:3, v/v/v) was the first solvent and methylene chloride-acetone (85:15, v/v) was the second. Specific areas corresponding to compactin or cyclopenol standards were scraped, collected, pulverized, and extracted twice with 1.0 ml acetonitrile. The extracts were combined, centrifuged, and the supernatant was assayed (HPTLC).

Immobilization and factors involved

A loofah sponge (Figure 2) was soaked in DW for 10 min, its core was removed and the outer layer was sliced into cubes ($\approx 1.0 \times 1.0 \times 1.5$ cm). After sterilization the cubes were again soaked in sterile SYEMB for 1 h and transferred to petri dishes, inoculated with a suspension of conidiospores, incubated (25°C) to the desired growth phase and then transferred to fermentation flasks or to a bioreactor. Factors affecting syntheses in the batch system examined were: growth phase of inoculum (age, h) used for immobilization, amount of loofah and incubation time. The flasks, containing 60.0 ml modified (A) MGPB and a specified amount of loofah cubes were inoculated, and incubated in a shaker water bath (25°C). The modified (A) MGPB had citrate and less glucose than the original MGPB (0.5% glucose and 0.01 M citrate, pH 4.0). Three growth phases were utilized and compared for compactin synthesis: conidiospore-suspension (0.3 ml containing 1×10^9 spores ml^{-1}) directly added to loofah cubes at zero time in the flasks; the second and third phases, the spores (same numbers) were inoculated, allowed to grow for 36 h (trophophase) or 106 h (idiophase), respectively. Each phase was used for immobilization on loofah and the desired medium was added. Free (not immobilized) spore-suspensions were utilized as controls and treated similarly. Assays of cell mass, pH, reducing sugars, and metabolites were conducted at intervals during each experiment. Different amounts of loofah were added to flasks containing 60.0 ml modified (A) MGPB, the flasks were incubated in a shaker water bath (25°C) for 12 days and compactin level as well as cell mass were determined. The specific compactin concentration was ascertained and reported as compactin (mg) total cell mass of loofah $(g)^{-1}$.

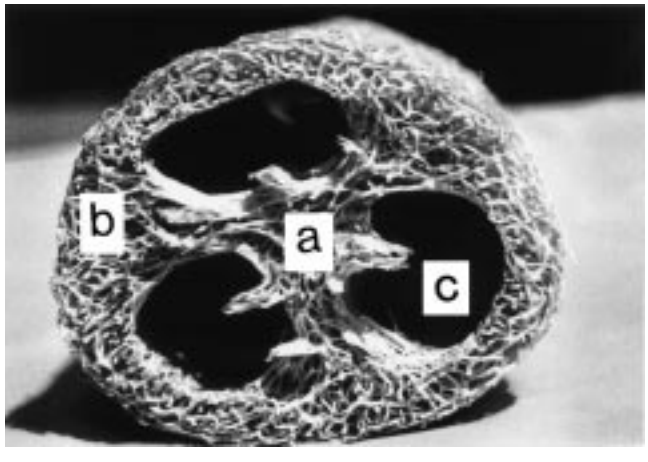

Figure 2 Photograph of loofah cross section consisting of: (a) the core; (b) the outer layer; and (c) the internal longitudinal channels.

Bioreactor system

Two sterile vertical Pyrex columns were used: No. I (27.0×3.3 cm) and No. II (44.5×7.5 cm), each with two bottom inlets (aire, medium) and one top outlet for exit port [28]. The total volume of each column (I and II) were 0.28 and 1.75 L with void volumes of 0.24 and 1.6 L, respectively. Column No. I was packed with loofah cubes (3.5 g dry weight) and No. II had the entire (whole) loofah. The immobilization was conducted as follows: six loofah cubes (6.5×6.0 cm) or the entire loofah, were placed in a 2-L covered beaker, sterilized, media inoculated, incubated for 24 h (25°C) and transferred to the column. Each column (I and/or II) was fed with modified (B) MGPB as feedstock (FS) containing ($g\ L^{-1}$ DW): malt extract, 30; glucose, 5; peptone, 1; and citric acid, 1.92 at the desired pH. Unless specified, sterile air was supplied to each column at 30 ml min^{-1} during fermentation, the system was operated in batch mode until the compactin level reached steady state. This was followed by continuous mode at which time the effects of dilution rates (h^{-1} = flow rate (ml h^{-1})/reactor void volume (ml)) were examined. The effluents were collected, assayed at intervals for compactin ($\mu g\ ml^{-1}$), productivity ($mg\ L^{-1}\ h^{-1}$) and reducing sugars (mg glucose equivalent ml^{-1}).

Scanning electron microscopy (SEM)

This was used to evaluate the loofah ultrastructure, the attachment of the conidiospores into-onto the loofah fibers and growth characteristics of the mold post-immobilization. Loofah specimens were fixed (3 min) in an ice bath using the Părducz [42] procedure, following specified conditions, mounted on aluminum stubs with silver paint and coated with a 400 Å mixture of gold-palladium (6:4, w/w) in a Hummer Sputter Coater (Alexandria, VA, USA) and viewed at 20 KeV using a Philips 505 SEM.

Analyses and statistics

The following methods were used for media and effluents: total reducing sugars [3], total carbohydrates [58] and growth kinetics [38] based on the Michaelis-Menten expression. The Monod constant was calculated from batch system data by fitting the linearized model expression to the data of average substrate weight and cell mass. All results were subjected to one-way analysis of variance [46], and unless otherwise stated, averages of three experiments reported.

Results and discussion

The crude extract of SWM, after 14 days growth of *P. cyclopium*, contained seven metabolites on TLC plates. The R_f values were: viridicatol, 0.09; cyclopenol, 0.16; viridicatin, 0.19; compactin, 0.35; cyclopenin, 0.44; unidentified

compound (A), 0.56; and unidentified compound (B), 0.68. Sequential solvent fractions of the crude extract on a silica gel column contained different metabolites in each fraction: the benzene-soluble fraction contained the unidentified compound (A); the tert-butyl methyl ether fraction had the seven metabolites; the ethyl acetate fraction contained five, but none of the A or B compounds; the acetone had viridicatol, and the acetonitrile yielded none. Since the seven metabolites were in the tert-butyl methyl ether fraction, further isolation of each metabolite was conducted on a large silica gel column (Figure 1) and 10-ml fractions were collected: Nos. 55–110 (550 ml total) had the metabolite (A); cyclopenin was in Nos. 135–205; compactin in Nos. 165–350; viridicatin detected with compactin in Nos. 165–250; cyclopenol in Nos. 420–650 and viridicatol in Nos. 470–650. The fractions containing compactin and viridicatin (Nos. 165–350) were combined, concentrated, and kept for 48 h at 24°C to allow formation of viridicatin crystals. The resulting mother liquor was seeded with pure compactin, to yield compactin crystals. Fractions 420–620 were combined, concentrated, kept at 25°C for 48 h and cyclopenol crystals separated out. The supernatant phase was concentrated and yellowish crystals were isolated which had a mass spectra of viridicatol. ^{1}HNMR analyses of the isolated compactin had spectral characteristics of the authentic compactin. Acetonitrile, methanol, and hexane solvents exhibited low UV absorbencies at 220 nm of 0.00, 0.26, and 0.08, respectively. Since the transition energies [43] in this region were higher for acetonitrile than for hexane or methanol, it was chosen to dissolve each pure metabolite. The absorption maximum for compactin in acetonitrile was 238 nm, with two minor peaks at 230 and 246 nm providing evidence for a bicyclic diene chromophore [37]. Absorbencies at 238 nm of acetonitrile containing different compactin levels (μg ml^{-1}), yielded a linear relationship and correlation of 0.9999 with a 0.5 μg ml^{-1} detection limit and 2.3% maximal error. In extracts of SWM, cyclopenin interfered with compactin and separation was necessary using HPTLC and the percent recovery was 48.5% ± 2.5. Spectra for cyclopenol (16.20 μg ml^{-1} acetonitrile) had only one maximum at 286 nm, confirming the finding of Cutler *et al* [8]. Absorbencies at 286 nm of different cyclopenol levels (1.0–50.0 μg ml^{-1}) had a straight line and correlation of 0.9999 with a detection limit of 1.0 μg ml^{-1} and 5.21% maximal error.

Compactin synthesis in submerged culture

Uniform growth of *P. cyclopium* in SYEMB, was noted after 10 days inccubation (density of 10 mg dry cells ml^{-1}). The levels of cell masses in GBSB and in MGPB were not different, but maximal after 8 and 6 days in MGPB and GBSB, respectively. In the GBSB, growth was not uniform, and for MGPB it was in pelleted form resulting in some limitations of nutrient transfer to the pellet core. The pH of all media used changed from 5.5 to 3.3 within 4 days incubation and reducing sugars in SYEMB increased 2.5-fold, after 21 days, due to invertase activity. However, total carbohydrates (Table 1) decreased after 21 days to 30, 4.2 and 118.4 mg ml^{-1} for MGPB, GBSB and SYEMB, respectively. The SYEMB did not support compactin synthesis (Table 1) possibly due to hydrolysis of sucrose to glucose and fructose which in turn may have suppressed the production of enzyme(s) for compactin synthesis. The organism in MGPB showed enhanced compactin production to ~1.6-fold that of GBSB at 12 days due to slow sugar utilization and formation of pellets which stressed the organism and induced higher compactin synthesis (60.1 μg compactin ml^{-1}). Drew and Wallis [13] reported that glucose served as an excellent substrate for growth, yet its rapid utilization and expression of secondary metabolites were mutually exclusive in many cases. Thus, limiting glucose favors secondary metabolism by *P. cyclopium*, which did not sporulate during incubation in submerged culture as evidenced microscopically. Furthermore, cyclopenol and cyclopenin synthesis were completely suppressed (not detected in extracts), contrary to the report of Schröder [50]. Georgiou and Shuler [26] stated that some conditions may be partially responsible for induction of differentiation, sporulation and secondary metabolites synthesis.

Increasing air flow from 0.5 to 3.0 (v v^{-1} min^{-1}), decreased the cell mass in MGPB during 12 days incubation and enhanced collision between mycelia and hyphal

Table 1 Effect of media on total carbohydrate (CHO) profiles (anthrone reagent) and on compactin (comp) during growth (days, 25°C) of *P. cyclopium* in submerged culture

Incubation (days)	Total CHO (mg ml^{-1}) and comp (μg ml^{-1}) ± SD in:					
	MGPB		GBSB		SYEMB	
	CHO	Comp	CHO	Comp	CHO	Comp
0	51.4 ± 1.9	0.00	89.4 ± 0.3	0.00	257.7 ± 8.0	0.0
2	48.7 ± 2.9	0.00	79.6 ± 0.6	0.00	234.4 ± 4.0	0.0
4	44.2 ± 1.0	29.80 ± 0.1	63.5 ± 1.8	10.56 ± 0.2	229.9 ± 7.8	0.0
6	40.4 ± 0.4	46.11 ± 0.2	53.6 ± 1.0	22.96 ± 0.7	210.2 ± 1.8	0.0
8	39.3 ± 1.0	54.21 ± 0.2	42.8 ± 1.0	30.91 ± 0.6	200.7 ± 2.7	0.0
10	37.2 ± 0.3	58.77 ± 0.1	33.7 ± 0.2	34.96 ± 0.5	190.2 ± 1.0	0.0
12	34.5 ± 1.0	60.10 ± 0.1	23.4 ± 0.5	37.75 ± 0.8	153.9 ± 4.3	0.0
15	31.4 ± 0.8	ND[a]	15.6 ± 0.6	ND	120.6 ± 1.4	0.0
18	30.1 ± 0.9	ND	7.6 ± 0.0	ND	120.3 ± 2.3	0.0
21	30.0 ± 1.0	60.11 ± 0.1	4.2 ± 0.0	38.02 ± 0.5	118.4 ± 1.0	0.0

[a]Not determined.

Table 2 Effect of different aeration levels on compactin (comp) synthesis during incubation of *P. cyclopium* in MGPB under submerged conditions at 25°C

Incubation (days)	Comp (μg ml^{-1}) ± SD and air levels (v v^{-1} min^{-1})		
	0.5	2.0	3.0
2	0.00	0.00	0.00
4	15.72 ± 0.39	37.40 ± 0.50	12.11 ± 0.32
6	37.61 ± 0.38	50.91 ± 0.25	34.92 ± 0.68
8	42.45 ± 0.42	60.11 ± 0.32	39.44 ± 0.51
10	45.01 ± 0.54	66.83 ± 0.43	41.25 ± 0.50
12	47.53 ± 0.81	70.00 ± 0.19	42.53 ± 0.41

Table 3 Effect of different glucose (glu) levels in MGPB on compactin (comp) synthesis during growth of *P. cyclopium* in submerged culture (25°C)

Incubation (days)	Comp (μg ml^{-1}) ± SD at glu level (%)		
	0.0	0.5	1.0
0	0.00	0.00	0.00
2	0.00	0.00	0.00
4	31.53 ± 0.83	39.18 ± 0.83	34.04 ± 0.57
6	46.22 ± 0.97	51.48 ± 0.94	55.01 ± 0.37
8	55.13 ± 1.34	63.54 ± 1.92	67.09 ± 1.13
10	58.06 ± 1.92	70.24 ± 1.12	74.12 ± 0.85
12	58.10 ± 0.56	73.31 ± 0.91	77.50 ± 1.33

Table 4 Effect of initial pH on compactin (comp) synthesis by *P. cyclopium* during submered growth in MGPB containing 0.1 M of citrate or succinate (pH 4.0) and succinate (pH 5.5)

Incubation (days)	Comp (μg ml^{-1}) ± SD at initial pH		
	pH 4.0		pH 5.5
	Citrate	Succinate	Succinate
0	0.00	0.00	0.00
2	11.62 ± 0.22	0.00	0.00
4	35.41 ± 0.82	31.13 ± 0.72	39.98 ± 0.94
6	50.90 ± 0.93	59.28 ± 1.01	54.44 ± 0.83
8	67.51 ± 0.42	76.24 ± 2.15	65.03 ± 0.27
10	84.79 ± 1.40	82.94 ± 2.01	73.75 ± 0.77
12	93.49 ± 2.29	92.91 ± 2.41	74.07 ± 1.98

Table 5 Effect of different molar citrate levels in MGPB (pH 4.0) on compactin (comp) synthesis by *P. cyclopium* during growth in submerged culture (25°C)

Incubation (days)	Comp (μg ml^{-1}) ± SD in media with citrate level (M)		
	0.01	0.05	0.10
0	0.00	0.00	0.00
2	8.40 ± 0.38	0.00	11.62 ± 0.22
4	30.15 ± 1.26	31.18 ± 1.31	35.41 ± 0.82
6	48.27 ± 2.30	50.66 ± 2.05	50.90 ± 0.93
8	86.69 ± 1.71	65.00 ± 1.38	67.51 ± 0.41
10	110.27 ± 1.36	84.29 ± 2.14	84.79 ± 2.72
12	118.48 ± 0.82	94.11 ± 3.37	93.49 ± 2.29

Table 6 Factors affecting maximal compactin formation and productivity by *P. cyclopium* during submerged growth in MGPB modified media (A) and (B). Media compositions are listed in Materials and Methods.

Factors examined	Compactin	
	Formed (mg L^{-1})	Productivity (mg L^{-1} h^{-1})
Air flow		
1 v v^{-1} min^{-1}	60.05	5.00
2 v v^{-1} min^{-1}	70.00	5.83
Modified medium (A), glucose (5.0 g L^{-1})	73.31	6.11
Modified medium (B), pH 4.0		
(0.10 M citrate)	93.49	7.79
(0.01 M citrate)	118.48	9.87

tips, thus metabolism was directed toward repair process rather than growth. Breakage of hyphal tips was detected microscopically at an air flow of 3.0. Again different aeration affected compactin production in MGPB under submerged conditions and higher values were noted at air level (v v^{-1} min^{-1}) of 2.0 as a function of incubation time (Table 2), and therefore this level of air flow was selected for subsequent experiments. During growth of MGPB and in the absence of added glucose, the media had a total reducing sugar of 20.4 mg ml^{-1} due to the presence of malt extract, but in all treatments, cell mass reached its highest level after 8 days. Compactin production in the same media, but with different glucose levels (Table 3), increased and continued thereafter to the 12th day, especially in the presence of 1% glucose. Unbuffered MGPB, with a pH of 4.0, 5.5 or 7.0, had no effect on the cell mass and compactin levels but the pH changed to 3.2 after 2 days incubation. When MGPB media were buffered with citrate or succinate (0.1 M) to yield pH 4.0 or 5.5, good growth was noted at pH 4.0 compared to 5.5 and the stationary phase was observed after 4 and 6 days at pH 4.0 and 5.5, respectively. Sporulation was also initiated, on the 2nd and the 4th days at both pHs and the distinctive green color of conidia was visible on the 5th day at pH 4.0, reaching maximal density (dark green) on the 7th day. Sugar metabolism was limited during the 12 days at pH 4.0 compared to pH 5.5 and compactin levels also increased at pH 4.0 in both citrate and succinate (Table 4). Therefore, citrate was added to MGPB (0.1, pH 4.0) in further experiments. The presence of citrate and Ca^{+2} stimulated sporulation and alkaloid synthesis by *P. cyclopium* [50]. Our results showed that sporulation occurred during compactin synthesis in MGPB buffered with the citrate, but no alkaloids were detected. Schröder [50] reported that citrate played a key role in pH stabilization, metal chelation and suppression of glycolysis, poss-

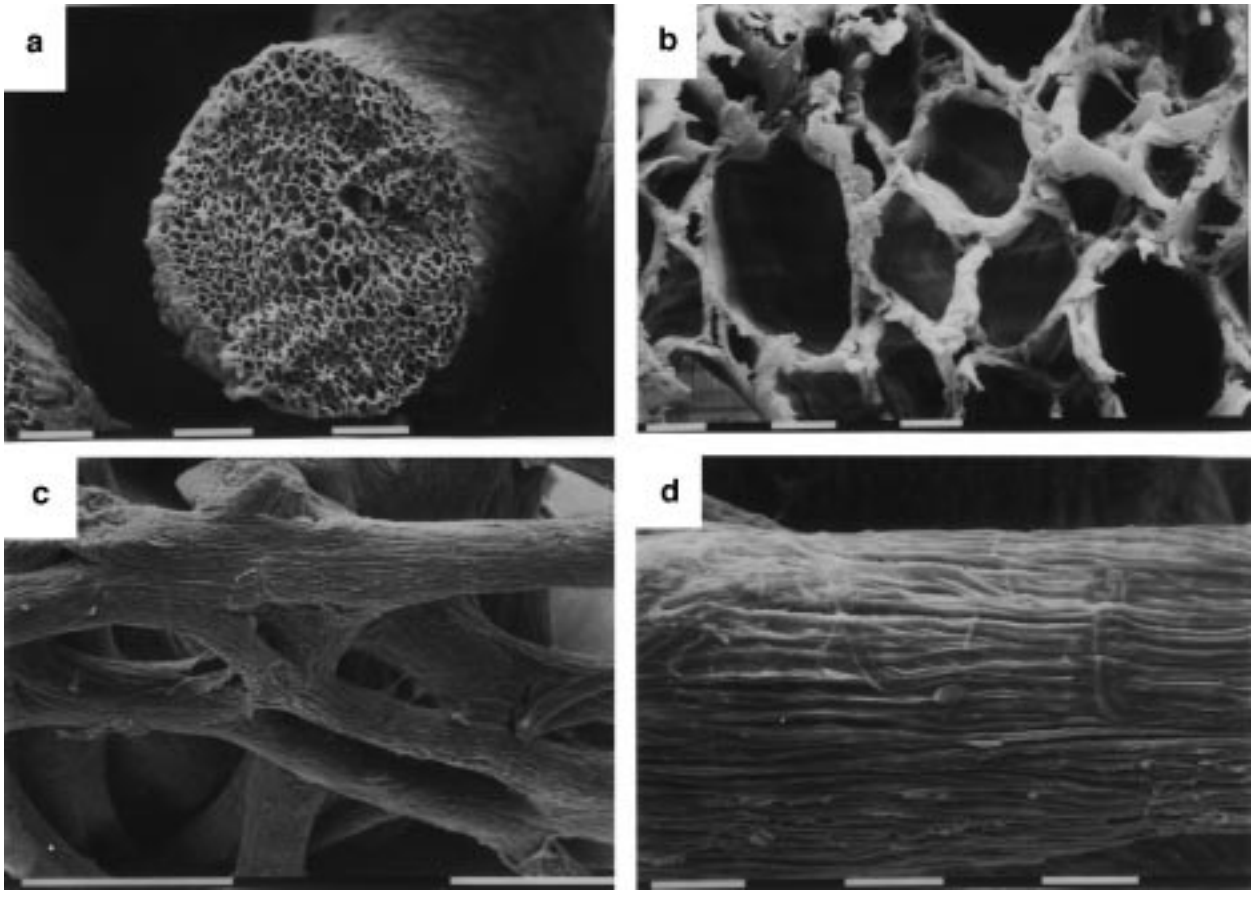

Figure 3 Scanning electron micrograph (SEM) of loofah prior to immobilization. (a) Cross section with different pore sizes and internal channels ranging from 5 to 25 μm; Bar = 0.1 mm and (b) = 10 μm; (c) network fibers with various directional changes (Bar = 1 mm); (d) surface channels on one fiber of the network (Bar = 0.1 mm).

ibly altering it to gluconeogenesis. Acetate, on the other hand, inhibited *P. cyclopium* growth, whereas citrate and succinate supported it and more growth was noted in MGPB with citrate reaching stationary phase within 4 days incubation with slight changes thereafter to the 12th day. Slow growth was observed in the presence of succinate to the 8th day followed by a decline thereafter, possibly due to lysis of cells as noted microscopically. We believe that citrate was utilized faster during the 2nd to 4th day as compared to succinate, leading to rapid growth, enhancement of cell specialization and sporulation. During growth, the pH of MGPB changed but was more stable in media containing succinate possibly due to ammonia liberation. However, no statistical difference was observed between compactin levels in media containing citrate or succinate at pH 4.0. At pH 5.5 for succinate during 12 days incubation compactin levels were lower. The molar concentrations of citrate in MGPB (buffered to pH 4.0) and under submerged culture conditions had no significant effects on cell mass or sugar utilization. Compactin reached a higher level in MGPB with 0.01 M citrate compared to 0.05 and 0.1 M citrate (Table 5) even though the cell mass was almost the same. The results of factors yielding maximal compactin synthesis in submerged culture (Table 6) indicated that compactin formed and productivity increased and that air flow, glucose (5.0 g L^{-1}) in modified (A) MGPB and initial pH of MGPB modified (B) with 0.01 M citrate yielded a higher level of compactin and its permeation rather than growth of the organism.

Immobilization

The SEM of loofah sponge prior to immobilization (Figure 3) showed the fiber network with different directional changes and internal pores as well as varied channel sizes. Longitudinal channels were noted on loofah fibers and the cross section had bundles of porous fibrils averaging 0.4 mm, and the diameter of single fibrils ranged from 5 μm to 25 μm. When the loofah was inoculated, the conidiospores were firmly attached to the surface channels, averaging 3.5 μm in diameter with irregular rough surfaces (Figure 4). Spore germination on loofah was detected within the first 10 h, followed by rapid growth (Figure 5) with hypha branching as well as septa formation, and colonies were present on the loofah surfaces within 24 h. The mycelia covered the entire loofah within 48–72 h, with sporulation and maximal conidiospore pigmentation after 96 h incubation. The loofah had large, rough channeled surfaces aiding attachment of spores and hyphae. The large internal spaces of loofah facilitated nutrient mass transfer, product synthesis and air movement during fermentation.

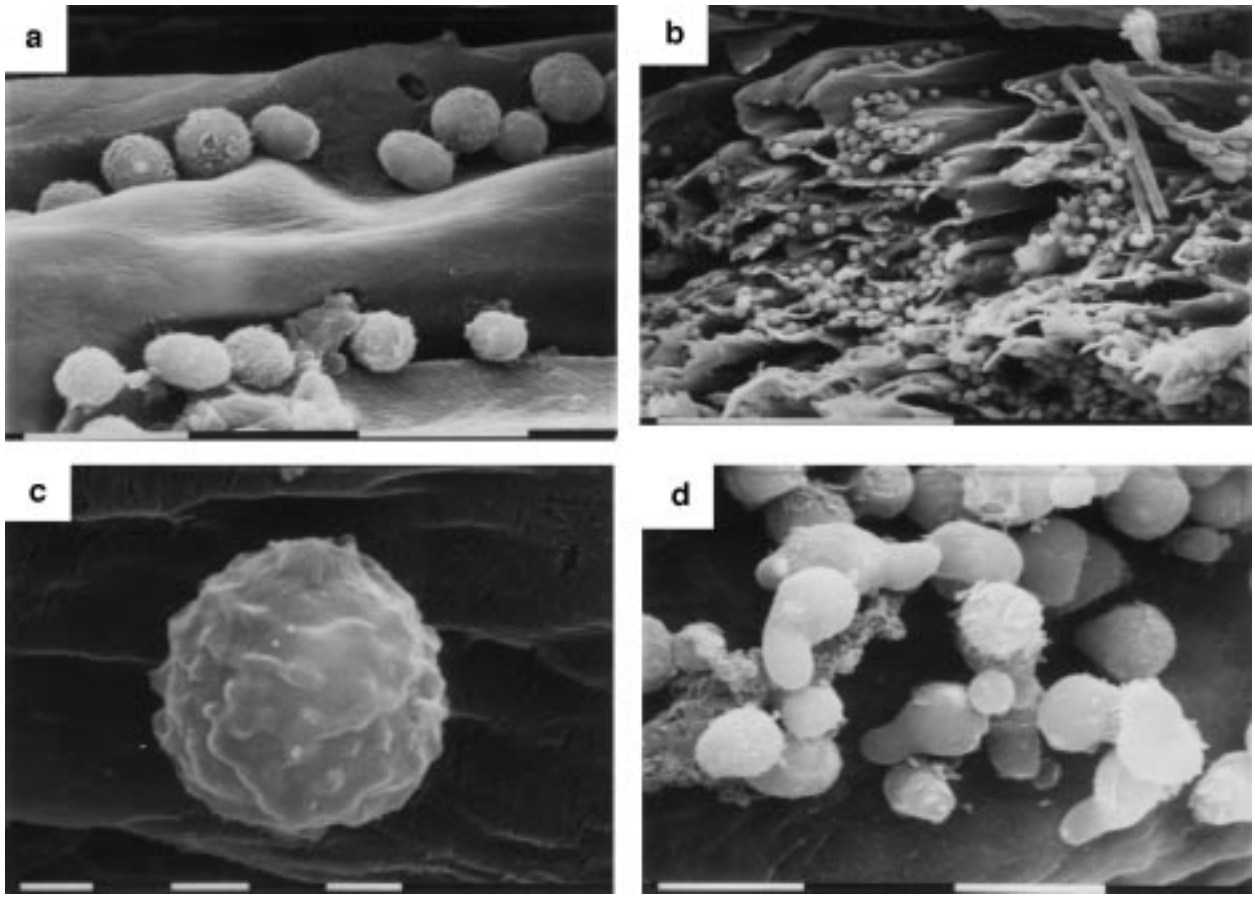

Figure 4 SEM for loofah (the solid carrier support) after inoculation with *P. cyclopium* conidiospores. (a) Spores attached to surface channels (Bar = 10 μm); (b) spores in cross section (Bar = 0.1 mm); (c) single spore, with rough surface (Bar = 1 μm); (d) germinated spores after 10 h incubation with average length of 3.75–4.75 μm (Bar = 10 μm).

Again, the loofah sponge is very light, durable with no apparent evidence of fiber hydrolysis following 2 months operation of the immobilized system. The morphological changes during submerged growth of immobilized *P. cyclopium* spores in MGPB, or in the modified (B) MGPB medium, showed the type of differentiation, the sporulation (48 h), pigmentation (72–96 h) as well as three growth phases: lag (germination), log (trophophase) and stationary (idiophase). These results confirmed those of Luckner [35].

Immobilized batch fermentation

At zero time, the initial cell mass in the control (no loofah) and in the flasks containing free spores immobilized on the loofah cubes were 0.71 and 0.61 mg dry wt ml^{-1} broth, respectively (Table 7). The trophophase cells (36 h) used for immobilization were in the log phase and resulted in a rapid biomass increase (26.68 mg dry wt ml^{-1}) within 2 days reaching maximal on the 12th day. Loofah cubes enhanced rapid fungal growth, provided protection for the hyphal tips during agitation and allowed for vigorous growth in internal spaces of the loofah cubes. However, the cells on the loofah surface were completely damaged and exhibited abnormal ends due to collision of cubes and the growth appeared toward the center of the loofah where the tips were protected. Idiophase-immobilized cells (106 h) showed a slight increase in cell mass during growth (0–12 days) possibly due to the presence of metabolites in the inoculum. Different ages of inocula were also used in the modified (B) MGPB shake culture with free spores (control) and showed no significant difference in sugar uptake (%) during the first 6 days of fermentation between control and free spores as well as between trophophase (36 h) and idiophase (106 h) immobilized-cell preparations. The level of compactin in shake cultures increased to maximal level after 14 days for all variables (Table 8). However, the highest value was noted in the control. The rapid production of compactin by immobilized idiophase cells during early growth (2–4 days) was probably due to its excretion into the medium from the inoculum and possible synthesis thereafter. Control flasks with free spores in modified (B) MGPB had more compactin than other flasks, possibly due to decreased cell mass and improved aeration. Cyclopenol was also detected in the immobilized idiophase cell preparations and excreted into the medium. Luckner [35] stated that cyclopenin, cyclopenol, viridicatin, and viridicatol were synthesized mainly in conidiospores and excreted into broth due to changes in spore permeability.

The higher the weight of loofah cubes per flask, the more

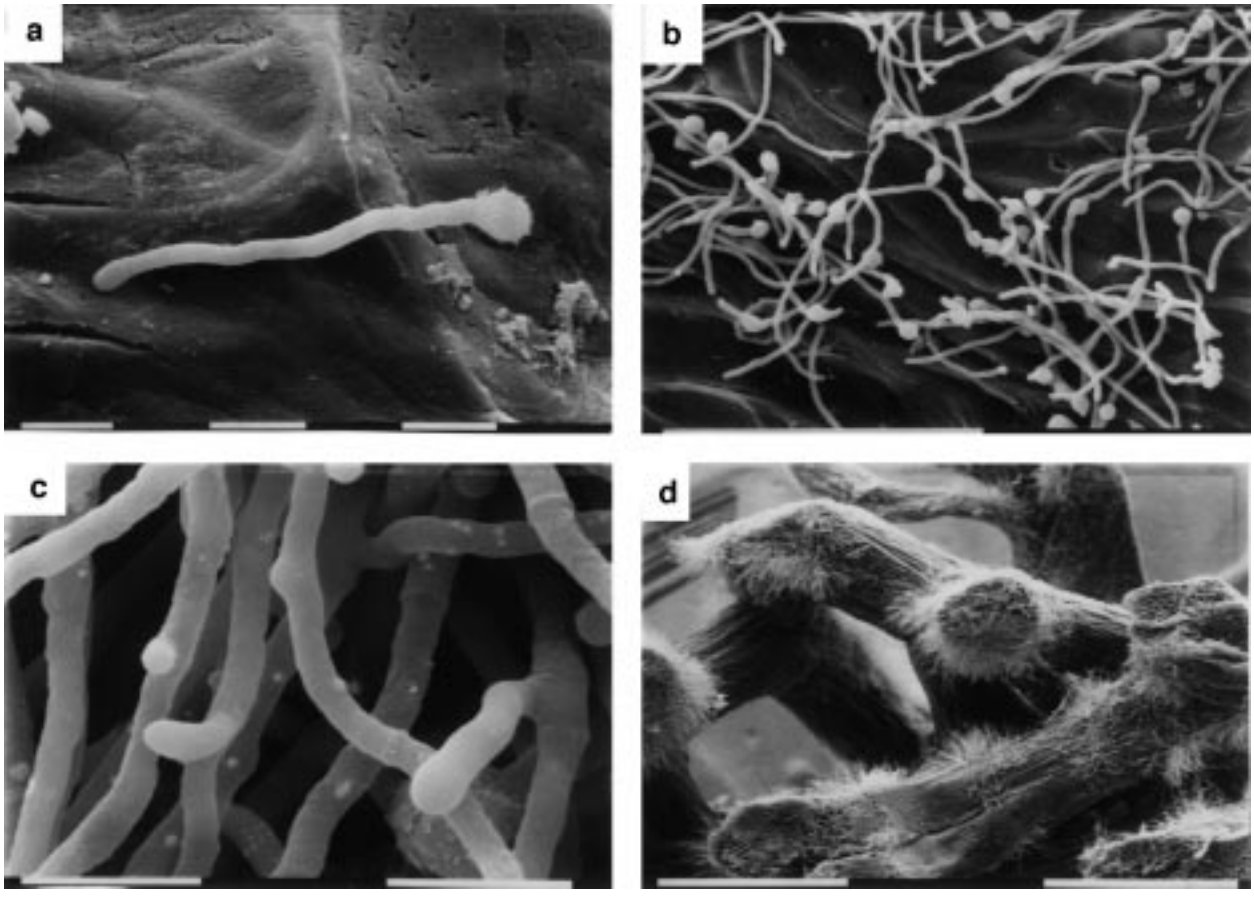

Figure 5 SEM for post-immobilized *P. cyclopium* on loofah. (a) Outgrowth of hyphae from one spore and smooth aspect of the cell wall after 18 h incubation (Bar = 10 μm); (b) hyphae on fiber surface during growth for 18 h (Bar = 0.1 mm); (c) directional changes of the mycelia after 24 h with least resistance, note the septa and branching formation (Bar = 10 μm); (d) loofah sponge after 24 h of fungal growth with dense colonies on loofah fibers (Bar = 10 mm).

Table 7 Cell mass (mg dry wt ml^{-1}) profiles for different ages of immobilized *P. cyclopium* cells during growth in modified (B) MGPB media with shaking at 25°C. The control had free cells

Incubation (days)	Cell mass (mg ml^{-1}) and conditions[a]			
	Control	Free spores	36 h	106 h
0	0.71	0.61	11.44	31.25
2	2.76	5.67	26.68	32.59
4	4.09	8.92	28.32	35.50
6	4.39	10.55	28.63	36.51
8	4.51	10.86	28.50	36.82
10	4.99	10.93	28.70	37.49
12	5.49	10.99	29.57	37.61

[a]Free spores added to MGPB containing loofah cubes at zero time; for others, spores added to loofah cubes saturated with SYEMB medium, incubated in petri dishes and immobilized preparations placed in Erlenmeyer flasks containing 60 ml modified (B) MGPB medium at zero time.

Table 8 Compactin (comp) synthesis using different growth phases for immobilization of *P. cyclopium* spores on loofah cubes using the modified (B) MGPB in shaken flasks and control had free cells (non-immobilized)

Incubation time (days)	Comp (μg ml^{-1}) ± SD and growth phase			
	Control	Free spores	Trophophase (36 h)	Idiophase (106 h)
0	0.00	0.00	0.00	0.00
2	23.51 ± 1.32	17.73 ± 1.31	19.70 ± 0.66	42.70 ± 2.34
4	61.81 ± 2.33	47.34 ± 2.45	34.24 ± 0.27	48.56 ± 2.85
6	84.73 ± 1.42	56.40 ± 2.25	49.32 ± 1.08	111.83 ± 3.34
8	112.90 ± 5.19	80.62 ± 2.62	77.95 ± 1.84	100.87 ± 3.60
10	130.01 ± 2.71	92.11 ± 2.63	108.33 ± 4.86	111.91 ± 3.55
12	157.85 ± 1.90	105.00 ± 1.44	111.00 ± 0.86	112.50 ± 2.14
14	170.05 ± 1.71	106.02 ± 1.83	111.07 ± 1.11	112.61 ± 1.96

cell mass formed due to increased surface area for spore attachment, germination and growth. The highest specific compactin concentration (38.4 mg compactin g^{-1} dry loofah) was noted when free cells (control) were used. The continuous system with loofah cubes (column I) using modified (B) MGPB media resulted in many operational problems after the 5th day of static fermentation due to blocking of air movement and nutrients into the column by heavy mold growth. Therefore, the larger column II, containing the entire loofah sponge, was subsequently used and resulted in an improved mass transfer of air and nutrients during operations of the bioreactor. The bioreactor sys-

tem was kept static for 7 days (batch-fermentation system without added fresh feedstock) and on that day, the compactin level was 78.6 μg ml^{-1} broth and remained constant on the 8th and 9th day (77.9 and 77.5 μg ml^{-1}, respectively). When the feedstock (modified (B) MGPB) was continuously fed to the system, at dilution rates of 0.12–1.35 h^{-1} (flow rates of 7.8–90.0 ml h^{-1}), the results (Table 9) indicated that an increased dilution rate enhanced reducing sugar from 10.6 to 19.6 mg (glucose equivalent ml^{-1}), and changed the pH from 3.3 to 4.0. The compactin productivities (mg L^{-1} h^{-1}) reached 23.04 at 0.45 dilution rate (30 ml h^{-1} flow rate) on day 4.4 and thereafter to the 30th day. However, compactin productivities declined to 13.82 mg L^{-1} day^{-1} at 1.35 dilution rate (90 ml h^{-1} flow rate) on the 33rd and 34th day, respectively. This decline was mostly due to rapid flow of the feedstock, and after 34 days operation the feedstock dilution rate was then reduced to 0.45 h^{-1} and the productivities, during the 36th–50th day, were constant (23.04 mg L^{-1} day^{-1}). The bioreactor system was operated continuously to the 51st day and turned off thereafter due to the massive fungal growth which limited air supply as well as nutrient transfer into the column, leading to plugging. These results showed that the immobilized *P. cyclopium*, in a continuous bioreactor system, improved the productivities over that noted for the submerged culture with air sparger. Application of immobilized microorganisms and carrier support used for the production of various secondary metabolites are of interest and have been reported by several investigators [6,19,33,41,48,55,56]: polyurethane [19,41,48], non-woven materials [55], and carrageenan [48]. Saucedo *et al* [48] stated that factors such as type of carrier, inoculum size, type of inoculum (spores or vegetative cells) and culture conditions (static, agitated or continuous) might lead to different fungal behavior and as a result, different data might be obtained. Some authors reported lower productivities using the reactor system than that using the conventional system and this is probably due to some limitations in mass transfer of substrate, product, oxygen, and dilution rate [9,32,47]. Therefore, new, effective immobilization methods for improving the efficiency of mass transfer will be of interest. In the present investigation the use of loofah sponge was evaluated as a novel carrier support for fungal cells and resulted in a higher compactin yield. The loofah is the fruit of the plant *Luffa aegyptiaca* from the *Cucurbitaceae* family [39]. Dried loofah has a fibrous, semi-rigid, sponge-like interior and is usually used as a gentle, abrasive washing sponge.

Table 9 Effect of dilution rate on compactin formed and productivity by immobilized *P. cyclopium* onto loofah in a bioreactor system (column II) using the modified (B) MGPB medium as feedstock. Results are averages of six samples for each analysis and data in brackets were operation times (days)

Dilution rate (h^{-1})	Reducing sugars[a]	pH value	Compactin level	
			Formed (μg ml^{-1})	Productivity (mg L^{-1} h^{-1})
0.12 (17.10)	10.60	3.3	55.00 ± 0.95	10.36
0.23 (8.88)	11.60	3.4	40.38 ± 1.28	14.59
0.45 (4.44)	12.67	3.6	32.10 ± 1.89	23.04
0.90 (2.22)	15.51	3.9	12.80 ± 0.57	18.43
1.35 (1.48)	19.57	4.0	6.54 ± 0.35	13.82

[a]mg glucose equivalent ml^{-1}.

Kinetics of product formed

Monod constant values of compactin synthesis in the submerged culture using modified (A) MGPB culture (air sparger, shake flask and trophophase immobilized cells) were calculated and were 60.0, 91.7 and 82.0, respectively. These values were high compared to those for primary metabolite production and are probably due to the fact that the precursors of compactin are not known at present. Microbial growth is a complex phenomenon but the overall growth can often be regarded as a single chemical reaction with a simple rate of expression. One of the simplest and most popular equations by Monod [38] was used for analyzing kinetics of fermentations, and for cell mass. Secondary metabolite synthesis also fits the Monod model.

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