ERGOSTEROL PRODUCTION UNDER OPTIMIZED CONDITIONS BY *PENICILLIUM CRUSTOSUM* THOM

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SUMMARY: Penicillium crustosum Thom was succesfully used for sterols production using clarified beet molasses (BM) as the sole carbon source. High yields of sterols and ergosterol were accumulated in the presence of some streol precursors (squalene or pyruvate). A cheep and economic fermentation medium that favoured maximum streol and ergostrerol yileds was found to contain the following ingredients (g/1): H_2SO_4-treated BM, 90; corn steep solid, 30; initial pH 7.0 and incubation period for 8 days. The GLC analyses revealed the formation of ergosterol (62.1%), stigmasterol (17.2%), fungisterol (16.4%), and lanosterol (4.3%).

Key Words: Penicillium crustosum, ergosterol.

INTRODUCTION

The production of sterols, more particularly ergosterol; the precursor of vitamin D, from was the aim of many investigators since the isolation of ergosterol from ergot (34). Several workers (10,12,29,32) reported that the sterol contents of a number of aspergilli and penicilla varied with different species and with the growth medium employed. Usually two or three different sterols form about 90% of the total sterols and the remaining 10% is represented by intermediate structures formed during synthesis of the predominant species (26).

In previous communications the authors found that *Penicillium crusosum* Thom was superior to the other molds for sterol production when being cultivated in beet molasses- containing medium. The sterol yields were markedly influenced by the clarification of beet molasses, composition and pH value of the fermentation medium as well as with the culture age. The effect of some physiological factors allowing better conversion of the beet molasses sugar into sterols by the tested fungus was also studied (14,15). These included beet molasses level, nitrogen requirements, role of K_2HPO_4 and $MgSO_4.7H_2O$, trace elements and time course production under optimal conditions. The present article deals with the role of some sterol precursors and the development of cheap culture medium.

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MATERIALS AND METHODS

Microorganism and Cultivation

Penicillium crustosum Thom was used throughout the present investigation. The culture was maintained on glucose-peptone slopes, then all fermentations received a 2% (v/v) spore suspension as inoculum of 5 day age. The beet molasses (BM) used throughout the present work was kindly supplied by the Delta Sugar Company, Egypt. The mould was growth in 100 ml portions of the medium dispensed in 250 ml Erlenmeyer flasks. The basal medium had the following composition (g/1): H_2SO_4 treated BM, 90; $(NH_4)_2SO_4$, 2.33; K_2HPO_4, 3.0; $MgSO_4.7H_2O$, 1.5; K_2SO_4, 0.11; 1ml of trace elements lacking $ZnSO_4$. 7H. The medium was adjusted to an initial pH of 7.0.

All nutrient solutions were sterilized by autoclaving for 15 min at 121°C. The sterilized media were inoculated and incubated at 30°± 1°C for different periods under stationary culture conditions. There after, the necessary analyses were model.

Analyses

The surface fungal mats were separated from the culture medium by centrifugation, washed with distilled water and dried at 60°C to constant weight. The mould dry weight was then estimated and the crude lipids were extracted (28), purified (11) and the lipid content was calculated, while the total sterols were estimated (3) in the unsaponified lipid fraction. The sterol mixture was analyzed by the Gas Liquid Chromatography of the acyl derivatives of the sterols present. The total ergosterol contents were estimated by ultra-violed absorption spectral analysis (33). The total nitrogen content and crude protein were determined by the micro Kjeldahl technique using BUCHI 321 digestion and distillation units.

The original, as well as the unassimilated, sugar contents of the BM were determined in the medium as glucose (5). Each treatment was carried out in triplicate and the result obtained throughout this work were the arithmetic mean.

RESULTS AND DISCUSSION

Role of some sterol precursors

The effect of the addition of some substances that may participitate directly or indirectly in the biosynthesis of sterols by fungi was investigated. The tested compounds were added separately to the fungal cultures (*in vivo*) and in other treatments supplemented to the fungal cell homogenate (*in vitro*) at 0.5 g/l level. The tested compounds were Na-acetate, Na-pruvate, and squalene (1,6,7). The results given in Table 1 revealed that the addition of acetate to the fermentation medium decreased the capacity of the tested fungus to assimilate the molasses sugars. This was correlated with the achievement of relatively lower growth yields. On the other hand, the biosynthesis of lipids other than the unsaponifiable fraction was accelerated in the presence of acetate. Strerols formation was also restricted under these conditions. Thus acetate seems to initiate the biosynthesis of the saponified lipids, mainly fatty acids and glycerides. Likewise, pyruvate acts positively on the formation of the saponified fraction of the fungus lipids, while the sugar uptake, growth or sterol yields appeared to be unaffected. In this respect, it has

Table 1: Growth ((D.wt.), lipids, unsaponified liqid fraction, total sterols and ergosterol contents of *Penicilium* crustosum thom for different incubation periods on modified basal medium supplemented with some sterol precursors (0.5 g/1).

Sterol precursor	Incubation period (day)	Sugar uptake (%)	Fungus D.wt (mg)	Total Lipids % to D.wt.	Unsaponified lipids		Total sterols		Total ergosterol	
					% to D.wt.	% to lipids	% to D.wt.	% to unsap.	%to D.wt.	% to sterol
None (control)	4	38.9	908	16.0	8.8	55.0	5.3	60.2	2.8	52.8
	6	62.8	1181	20.7	11.0	53.1	8.6	78.2	5.1	59.3
	8	70.3	1159	21.9	12.0	54.8	9.7	80.8	7.0	7.22
	10	75.6	923	23.5	10.3	43.8	8.2	79.6	3.9	47.6
Na-acetate	4	30.4	798	21.9	8.3	37.9	6.8	81.9	4.4	64.7
	6	48.6	903	27.4	9.7	35.4	8.8	90.7	5.9	67.0
	8	52.6	1166	26.5	11.9	44.9	9.0	75.6	3.3	36.7
	10	62.2	1184	24.5	10.0	40.8	7.8	78.0	2.7	34.6
Na-pyruvate	4	36.0	1010	18.3	8.2	44.8	5.8	68.3	2.4	41.4
	6	62.1	1213	24.4	11.1	45.5	9.0	81.1	5.8	64.4
	8	73.0	1186	26.1	11.8	45.2	9.3	78.8	7.4	79.6
	10	75.4	966	27.6	10.7	38.8	8.6	80.4	4.1	47.7
Squalene	4	60.7	1302	24.1	12.6	52.3	8.1	64.3	4.9	60.5
	6	63.1	1369	26.2	14.7	5.1	9.3	63.3	6.7	72.0
	8	64.6	1384	28.1	18.8	66.9	9.8	52.1	7.5	76.5
	10	66.1	1165	25.2	13.0	51.6	8.7	66.9	5.4	62.1

Table 2: Total lipids, unsaponified lipid fraction, total sterols and ergosterol contents of the tested fungal cell homogenate suspended in phosphate buffer as influenced with some sterol precursors.

Supplement (0.25 g/5 g cell homogenate)	Total lipids % to D.wt.	Unsaponified lipid % to D.wt.	Total sterols % to D.wt.	Total ergosterol % to D.wt.
None (control)	22.0	11.3	8.2	5.5
Na-acetate	25.8	9.1	6.5	3.6
Na-pyruvate	25.9	12.9	9.6	5.8
Squalene	28.4	19.8	11.5	8.6

been shown that ergosterol can be formed acetate (31), or squalene (4). While it was reported (7) that acetate stimulated the unsaponifiable content of *Sacchoromyces fermentatii* with out affecting the sterol fraction. But, in accordance with the present results, other workers (22) found that acetate is incorporated in strerols of *Dictyostelium discoideum* at first rapidly, dropped, then increased slightly.

Additionally, evidence has been shown that equalene supplement stimulated the assimilation of BM sugars together with the production of relatively high fungus biomass yields. Moreover, squalene accelerated the biosynthesis of the unsaponified lipids, particularly the sterols fraction. Ergosterol contributed the major part of the sterol content. These findings are in hormony with the results of many reports elucidating the basic role of squalence in sterol biosynthesis (2,4,25,32).

The analysis of variance indicated that the variations with the tested sterol precursors, for different incubation periods, in the content of total lipids, unsaponified lipids, and total sterols as well as total ergosterol were significant ($F=6.84, 9.46, 10.42$, and 5.30 respectively; $P<0.05$).

The role of Na-acetate, Na-pyruvate and squalene was also studied under controlled conditions to avoid the broad metabolic patterns of the growing fungal cells frequently resulting, when the fermentation process was conducted in culture media. This was approached by collecting the fungal mats grown on the basal medium for 4 days. The harvested mats were collected aseptically and washed with sterile distilled water. Each 5.0 g fresh fungal weight were mixed with 5 g sterile acid clean sand, then ground well and examined microscopically to assure the destruction of the fungal cell walls and the homogeneity of the preparation. The fungal cell homogenates (5.0 g) were suspended in 50 ml aliquots of phosphate buffer of pH 6.98 containing 0.25 g of the tested substance 1% glucose and 0.01% Na-azide (to contamination). The mixture was then incubated for 4 days at 30°C under stationary culture condition, and the necessary analyses were carried out at the end of this period. A parallel treatment (control) was conducted. In this respect, it was stated (17) that a cell-free system from *Saccharomyces cerevisiae* was capable of incorporating acetate into fatty acid and unsaponifiable fraction. The result of this study (Table 2) assessed the stimulatory action of acetate, pyruvate and squalene on lipid accumulation. However, the lipid groups affected positively under this condition, proved to be dependent on the type of the additive employed. The unsaponified lipids were enhanced in the presence of pyruvate or squalene where in total sterols particularly ergosterol were produced in relatively high levels. On the other hand, acetate encouraged only the formation of the saponified lipids e.g. glycerides.

The analysis of variance indicated that the variations with the tested sterol precursors, under controlled conditions, in the content of total lipids, unsaponified lipids and total sterols as well as total ergosterol were significant ($F=10.54, 19.4, 16.7$ and 9.40 respectively; $P<0.05$).

Development of cheap culture medium

In an attempt to develop a cheap culture medium clarified beet molasses (9%) fortified with 3% corn steep solid (with nitrogen equivalent to 2.33% $(NH_4)_2SO_4$) was employed for the cultivation of the tested fungus. This formulation was successfully used by the experimental fungus to produce high fermentation yields, comparable to those obtained when the fungus was cultivated under the physiological conditions previously optimized. Thus, after 8 days of incubation (Table 3), the most vigrous growth (1623 mg/100 ml) as well as the highest total sterols (9.9%) and ergosterol (6.1%) were attained. It is safe to conclude that the composition of this cheap natural medium fulfield all the nutritional requirements of the tested fungus which allowed the production of the highest

Table 3: *Penicillium crustosum* Thom, growth, growth (D.wt.), protein, total lipids, unsaponified lipid fraction, total sterols and ergosterol contents under cheap culture conditions.

Incubation period (days)	Sugar uptake (%)	Fungus D.wt. mg	Protein % to D.wt.	Total Lipids		Unsaponified lipids		Total sterols		Total ergosterols	
				mg	% to D.wt.	mg	% to D.wt.	mg	%to D.wt.	mg	% to D.wt.
2	20.9	626	24.9	134.0	21.4	78.9	12.6	48.8	7.8	23.8	3.8
4	45.9	862	22.8	202.6	23.5	91.7	10.6	72.4	8.4	36.2	4.2
6	46.8	1283	21.7	342.6	26.7	120.7	9.4	81.9	6.4	53.4	4.2
8	65.0	1623	21.9	490.1	30.2	272.7	16.8	160.0	9.9	98.5	6.1
10	65.9	1328	20.6	425.0	32.0	223.1	16.8	132.1	9.9	66.3	5.0

sterol and ergosterol yields encountered in the present work. In this connection, some workers (6,7) found that molasses-corn steep liquor is an inexpensive medium but supports good growts and sterol production. It was also found that a cheap culture medium consisting of 8% treated molasses and 1% corn steep liquor favoured maximum growth and total lipids yields of *Fusarium oxysporum* (24). The stimulatory effect of the phenylalanine present in corn steep liquor on mycelial growth, total proteins and sterols produced by *Aspergillus fumigatus* was also demonstrated (8). Other workers have explained the stimulatory effects of corn steep liquor on growth and lipid production by fungi as due to the presence of certain sugars, amino acids, keto acids and vitamins (23,27).

The analysis of variance indicated that the variations with the incubation periods, under cheap culture conditions, in the content of total lipids, unsaponified lipids, and total sterols as well as total ergosterol were significant ($F=24.61$, 46.19, 16.40, and 12.40 respectively $P<0.05$). Gas liquid chromatogrophy analyses of the sterols.

The GLC analyses of the sterol fractions synthesized by the fungus grown in a cheap culture medium (clarified BM+corn steep solid) for 8 days (maximal mould activities) revealed the presence of ergosterol, as the main sterol (62.1% of the total sterols), its 5,22-dhydroderivative namely fungisterol (16.4%), and stigmasterol (17.2%) as well as minor amounts (4.2%) of lanosterol. Ergosterol was identified as the major sterol of many moulds and yeasts. There is a quite number of literatures referring to its occurrence in a variety of microorganisms (2,9,13,21). Fungisterol accompained ergosterol in ergot. It also occurred in many microrganisms (13,30,35). Stigmasterol and ergosterol as sterols with a double bond in side chain were found together in the protoplast of some yeasts where they are considerably more stable than those with a saturated side chain, cholesterol, sitosterol (18). Ergosterol stigmasterol and fungisterol from *Penicillium notatum* were isolated (29). Many workers (19,20) reported that some fungi posses sterols of the stigmastane. Lanosterol, the initial cyclized precursor of ergosterol, was identified as a minor component of the sterols of many fungi by several workers (16,25,32).

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