Sci. J. Fac. Sci. Monoufia Univ., Vol. II. 1988.

NITROGEN SOURCES, VITAMINS, INDOLES AND GIBBERELLIC ACID AS FACTORS INFLUENCING THE PRODUCTION OF LIPASE BY THE PHYLLOSPHERIC YEAST TORULOPSIS DOMERQUII

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ABSTRACT

An attempt was made to evaluate lipase productivity by Torulopsis domerquii when its culture growth medium was supplemented with equivalent amounts of nitrogen from different nitrogen sources, 10, 50, 100 or 200 ppm of water soluble vitamins; indole acetic acid (IAA), indole propionic acid(IPA) and indole butyric acid (IBA) or gibberellic acid (GA_3).

Results have shown that $(NH_4)H_2PO_4$ was the best inorganic nitrogen source. The use of equivalent amounts of nitrogen from various amino acids to that present in 0.2 % $NaNO_3$ revealed the importance of these sources on lipase production. A mean value of 10000 µg/ml was produced when L-lysine was the nitrogen source but no lipase was produced at all when each of L-histidine, Dl-aspartic acid, L-cysteine or Dl-isoleucine was singly supplied. Other amino acids either stimulated lipase production or partially inhibited its production.

Supplementing 10, 50, 100 or 200 ppm of water soluble vitamins and yeast extract resulted in stimulatory effects. There was an optimal concentration for each vitamin for maximal lipase production. Maximum amounts of lipase were produced on using 10 ppm cyanocobalamin and folic acid and 50 ppm of each of PAPA, biotin and ascorbic acid. 50 ppm. and higher concentrations of pyridoxine HCL were found inhibitory for lipase production.

Indole compounds and gebberellic acid showed a general stimulatory effect on lipase production. Fifty ppm. IPA and IBA and GA_3 and 100 IAA were found to be the best concentrations for lipase production by T. domerquii.

Discussion of the nature of the observed stimulation or inhibition has been given.

INTRODUCTION

Production of lipase by microorganisms is affected by many factors of which are nitrogen sources, vitamins, indoles or gibberellins. The nature of nitrogen source in a culture medium affects lipase production; the effects is either stimulatory (Weisbrodt, 1927; Naylor et al., 1930 and Elwan et al., 1982) or inhibitory (Cutchins et al, 1952; Alford & pierce, 1963, & Elwan et al., 1982). The supplementation of amino acids to culture media caused no marked increase in lipase production (Peters & Nelson, 1948) or yielded moderate amounts of lipase (Nelson, 1953). The production of lipase by *Pseudomones flourescens* increased when aspartic acid was used as a nitrogen source and olive oil as the carbon source (Cutchins et al., 1952). Nashif and Nelson (1953) found that leucine , isoleucine and valine caused an increase in lipase production by *Ps. fragi.* Elwan et al., (1982) found that arginine was optimal for lipase production by *Penicillium lanosum.* Also, Alford and Pierce (1963) and Elwan et al., (1982) noticed maximal lipase production by *Ps. fragi.* and *P.lanosum* in peptone medium respectively. Some workers found that vitamins had no lipase production (Peters & Nelson 1948; Nelson, 1953 & Alford & Pierce, 1963). Other workers showed that the addition of vitamins or yeast extract stimulated lipase production (Peters & Nelson, 1952; Alford et al., 1964 & Elwan et al., 1982). Although indoles and gibberellins were reported to be produced by many microorganisms yet work on their potential relation to lipase production seems lacking as far as the available literature shows. Only Elwan et al., (1982) recorded an increae in lipase production when applying indoles and low concentration of gebberellic

acid. High concentration of gibberellins was inhibitory.

MATERIALS and METHODS

Organism:

Torulopsis domerquii which was found the most potent lipolytic yeast isolated from the phyllosphere of cotton plant (Desouky, 1976) has been used in this work.

Lipase assay:

It was carried out by the tributyrin cup-plate clear zone technique of Lawrence et al., (1967) as deviced by Elwan et al., (1977).

Media:

Gorodkwa medium (Lodder, 1970) was used for preparation of yeast suspension for inoculation. It contains 0.25 % glucose, 1 % peptone and 0.5 % sodium chloride. 2 % agar was used for solidification and autoclaved for 15 minutes at 1.5 atmos.

The medium used for assessment of lipase production was DYC (Dox-yeast extract-corn oil medium). it contained the inorganic components of Dox's medium viz $NaNO_3$, 0.2 % , K_2HPO_4, 0.1 % $MgSO_4$. $7H_20$, 0.05 %; KCI, 0.05 % and traces of Fe SO_4. $7H_20$, dissolved in distilled water and mixed with emulsified 0.2 % corm oil (instead of sucrose in normal Dox) DC corn oil medium 0.1 % yeast extract was supplied in case of DYC medium.

Inoculum size:

The growth of 48 hours was harvested aseptically and suspended in known volume of sterile physiological saline solution. 1.0 ml. of homogenised stock suspension containing about 40 milions cells was used as an inoculum.

Cell-free culture filtrate:

This was obtained by passing the culture filtrate through Whatman No. 1 filter paper using Buchner, the filtrate was centrifuged at 4000-5000 r.p.m. for 15 minutes and the supernatant was then passed through G_6 sintered glass funnel. The filtrates were checked microscopically and streaked on nutrient and Gorodkowa agar media. Sterile filtrates only were assayed.

Effect of various nitrogen sources:

To $NaNO_3$-free DYC medium 329.4 mg nitrogen (equivalent to nitrogen of 0.2 % $NaNO_3$) of the inorganic nitrogen supplements represented by NH_4NO_3 ; $(NH_4)_2 SO_4$; $HN_4H_2 PO_4$ & $(NH_4)_2 HPO_4$ and organic nitrogen supplements represented by urea and 20 amino acids were added. Effect of 0.2 % peptone (of 268.6 mg nitrogen content) as an organic nitrogen supplement in DYC was also tried. In all cases pH was adjusted at 5.8 using citrate buffer and incubation was made at 30°C for 5 days.

Effect of water soluble vitamins, yeast extract indole compunds and gibberellic acid:

The vitamins (Prolabo production) riboflavin, pyridoxine, pantothenic acid, folic acid, cyanocobalamin, biotin , inositol p-aminobenzoic acid (PABA) nicotinic acid, yeast extract (Dif co), the indole compounds (B.D.H. product) , indole-3-yl acetic acid, indole-yl-propionic acid and indole-yl-butyric acid and gibberellic acid (GA_3 B.D.H. product) were supplied singly to ingredients of DC medium at concentration of 10, 50, 100 or 200 ppm. control was DC medium receiving no supplement. Inoculation, incubation and lipase assay were made as mentioned above.

RESULTS

Linearity of the standard curve used in assaying lipase is shown in Fig. 1. Results showed an increase in values of clear zone diameter by increase of enzyme concentration used. The linearity range was from 0.01785 to 10000 µg/ml. this is relatively wide range of linearity enough for extrapolation of the clear zone values of 9.5 to 19 mm. Admittedly, the approximation of the diameters to the nearest 0.5 mm make the determinations semiquantitative and it is important to consider the slope of the standard curve particularly in determining optimal conditions of production where precision in the amount obtained or its range value is required.

Production of lipase was evidently affected by the nature of the nitrogen source. Results are given in Table (1) . Maximum lipase ($9.75^{+4.8750}_{-2.4375}$ µg/ml.) was obtained when $NH_4H_2PO_4$ was the nitrogen source. The least amount of lipase ($1.219^{+0.6095}_{-0.3095}$ µg/ml) was obtained with ammonium sulphate and urea. Ammonium nitrate, dibasic ammonium phosphate and peptone producted equal ammounts of lipase namely $4.875^{+2.4375}_{-1.2185}$ µg/ml). Ranges of lipase production were 7.3125- 14.625 for $NH_4H_2PO_4$ on one side and 3.6565- 7.3125 for NH_4NO_3, $(NH_4)_2HPO_4$ and peptone on the other side. it could thus be said that lipase production was more induced by $NH_4H_2PO_4$ as compared to the other compounds.

Results Table(1) have also shown maximum lipase production (10000 µg/ml $^{+}$-2500) was obtained when L-lysine was the nitrogen source in DYC medium. On the other hand, when any of the amino acids L-histidine, DL-aspartic acid,

Table (1) : Effect of supplying amounts of nitrogen equivalent to $NaNO_3$ nitrogen on lipase production by *Torulopsis dómerquii* grown in sugar and NO_3 free Dox yeast extract-corn oil (DYC) medium at 30 ℃ for five days and pH 5.8 as determined by the TCZ assay. Errors given correspond to 0.5 mm clear zone diameters supposedly relative to the slope line of the standard curve.

Nitrogen source	Lipase production (µg/ml)		
Control (with N_aNO_3)	2.438	+ 1.2185	- 0.6095
NH_4NO_3	4.875	+ 2.4375	- 1.2185
$(NH_4)_2\ SO_4$	1.219	+ 0.6095	- 0.3095
$NH_4H_2PO_4$	9.750	+ 4.8750	- 2.4375
$(NH_4)_2HPO_4$	4.875	+ 2.4375	- 1.2185
Urea	1.219	+ 0.6095	- 0.3095
Peptone	4.875	+ 2.4375	- 1.2185
DL-proline	0.3	+ 0.1500	- 0.075
L-tryptophan	0.0	-	-
L-phenylalanine	0.0375	+ 0.0187	- 0.0095
L-histidine	0	-	-
L-lysine	10000.	-	- 2500
L-arginine	39.0625	+ 15.4687	- 9.78125
L-glutamine	156.250	+ 78.125	-39.0625
L-glutamic acid	78.125	+ 39.0625	-15.4687
L-asparagine	0.15	+ 0.0750	- 0.0375
L-methionine	0.15	+ 0.0750	- 0.0375
DL-aspartic acid	0	-	-
L-cysteine	0	-	-
Dl-threonine	0.15	+ 0.0750	- 0.0375
L-serine	0.3	+ 0.1500	- 0.075
DL-isoleucine	0	-	-
L-leucine	0.15	+ 0.075	- 0.0375
DL-Valine	0.3	+ 0.1500	- 0.075
Glycine	0.3	+ 0.1500	- 0.075
L-alanine	2.438	+ 1.2185	- 0.6095
Dl-tyrosine	0.0375	+ 0.0187	- 0.0095

L-cysteine and DL-isoleucine was the nitrogen source, no lipase was produced by *T. domerquii* in this medium. Compared to the mean value of the control, mean values of DL-proline, Dl-valine, L-serine and glycine showed 87.5% inhibition, whereas L-aparagine, L-methionine, DL-threonine or L-leucine showed 93.7 % inhibition. L-lysine, L-glutamine, L-glutamic acid or L-arginine caused an increase of about 4102. 64, 32 and 16 folds of control respectively.

With regard to the effect of vitamins on lipase production by *T. domerqui*, results are shown in Fig. (2) Acorbic, folic acid pantothenic acid, inositol, biotin and paraamino benzoic acid (PABA) stimulated lipase production at all supplemented concentrations. Ribofeavin, cyanocobalamin and folic acid gave more or less similar pattern of effect - represented by maximum stimulation at level of 10 ppm supplementation. In other vitamins optimal concentrations were reported to be 50 ppm (PABA, biotin, pantothenic acid or ascorbic acid) or 100 ppm (inositol). Yeast extract supposedly supplying a mixture of vitamins gave a pattern not represented by the singly supplied stimulatory vitamins. Nicotinic acid and pyridoxine HCl exerted inhibition of lipase production by *T. domerquii* at 10 ppm and higher concentrations.

With regad to indole compounds and gibberellic acid, results(Fig. 3) indicated that 10 and 50 ppm of each of the indolic compounds and gibberellic acid resulted in a stimulatory effect. Fifty ppm supplementation was found optimal for maximum lipase production by indole propionic, indole butyric and gibberellic acids, the optimal for indole acetic acid was 100 ppm. With the exception of gibberellic acid , 200 ppm supplementation showed inhibition (in case of indole propionic acid) or caused no effect (in case of indole acetic acid or indole butyric acid) on lipase production by *T. domerquii*.

DISCUSSION

The technique used in this investigation was so called tributyrin cup-plate clearing zone assay (TCZ) deviced by Elwan et al., (1977) from the technique described by Lawrence et al., (1967). Standard curve of Merck pancreatic lipase was constructed from quantities of lipase that have been determined. Although this technique was helpful in screening the potency of lipase production among isolates and also in detecting the optimal conditions required for lipase activity, yet it is after all semiquantitative. For the present purpose, it was satisfactory. However, approximation of the diameters of the clearing zone to the nearest 0.5 mm (inveitable in applying the techmique) implied expressing results in range values rather than absolute ones particularly when linearity extended in the standard curve between a fraction of microgram and 10000 micrograms/ml. This makes important considering the errors due to approximation as determined from the standard curve. The precission would be improved by magnifying the clear - zones and making measurements with a narrower scale.

With regard to the inorganic nitrogen source, it could be said that lipase was more induced by $NH_4H_2PO_4$ as compared to the other compounds. However, it would be diffcult to interpret the different effects of $NH_4H_2PO_4$ and $(NH_4)_2\ HPO_4$ since equivalent nitrogen was supplied to the medium and the pH of the medium was adjusted at 5.8. The apparent differnce is the number of ammonium ions in both cases and not the amount of nitrogen. Fewer molecules of $(NH_4)_2\ HPO_4$ supply nitrogen equivalent to that

of $NH_4H_2PO_4$. This means that phosphate ions (fewer-in the former than in the latter in weights of equivalent nitrogen) would interfere being more in supplied $NH_4H_2PO_4$ (the optimal for production) than in $(NH_4)_2HPO_4$. Thus the difference in effect might be correlated with or at least affected by the phosphate ions. In favour of this, is the difference between lipase production in case of $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$, the former being more (Table 1).

The role of NH_4^+ as an inducer to more lipase production could be known from the difference in effect between $NaNo_3$ and NH_4NO_3. Supply of half the amount of N in the form of NH_4^+ induced ragne of production from 1.8285 - 3.6565 (for $NaNO_3$) to 3.6565 - 7.3125 (for NH_4NO_3) indicating a stimulatory effect of NH_4^+ on lipase production by *T. domerquii*. In the present investigation NO_3 nitrogen induced the least amount of lipase which is contradictory to the behaviour of *Penicillium lanosum* (Elwan et al., 1982) where $NaNo_3$ stimulated maximal lipase production.

Although NH_4^+ was found more favourable for lipase production, yet this ion in the form of $(NH_4)_2SO_4$ was found less favourable as compared to NO_3^- in the form of $NaNO_3$. This could be justified by keeping in mind the amphotericity of cell protein at pH 5.8 where NO_3^- would have affinity to NH_3^+ of the protein; it is meant to say that difference in effect would be due to difference in charge and eventually the ions absorbability. All these aspects would be correlated to growth which is supposedly correlated with enzyme production and this admittedly needs further work.

Equivalent amounts of nitrogen from 20 amino acids resulted in inhibition, stimulation or no effect on lipase production by *P. lanosum* (Elwan et al., 1982) isoleucine, leucine and valine inhibited lipase production by *T. domerquii* wheras these amino acids caused an increase in lipase production by *Pseudomonas fragi* (Nashif and Nelson, 1953). The stimulatory effect due to L-lysine, L-glutamine, L-glutamic acid and arginine was in accordance to their effect on Ps. *fragi* as shown by Alford & Pierce, (1963) and contradictory to their effect on *P. lanosum* (Elwan et al., 1982). Alford and Pierce (1963) reported stimulatory effect on lipase production due to aspartic acid a case different from the effect of this amino acid on *T. domerquii*. Cystiene HCl caused no marked increase in case of *Mycotorula lipolytica* (Peters and Nelson, 1948) in opposite to its stopping of lipase production by *T.domerquii*. However, in some cases the effect of the amino acid does not change in various microorganisms; similar effects of alanine on lipase production were obtained in case of *Ps. aeruginosa* (sierra, 1957) and the present strain of *P.domerquii* .

Maximum production of lipase due to supplying L-lysine (6 C and 2 N) as compared to stimulation due to L-arginine (6 C and 4 N) and complete inhibition due to L-histidine (6 C and 3 N), all basic amino acids, would reflect the role of the form in which N is combined [2 (NH_2 in lysine; $2NH_2$ and 2(NH) in arginine; one (NH_2), one (NH) and one (N) in histidine].

Vitamins, indoles and gibberellic acid are considered important factors affecting lipase production. An observation which stimulated interest is the role of para amino

benzoic acid (PABA) in lipase production. This substance got its optimal effect when supplied at 50 ppm. It is known that PABA is a part required for the synthesis of the vitamin folic acid. It is a component of this vitamin. PABA by itself is an essential metabolite required for growth of many microorganisms. PABA was found in some yeasts to be formed and excreted in the medium (Lampen et al., 1945 c.f. Thiman, 1963). Folic acid is also synthesised by the majority of microbes in one or another form (Burkholder et al., 1945; Van Lanen and Tanner, 1948 c.f. Thiman, 1963). Yeasts may contain about 80 mg folic acid per gram dry weight(Thiman, 1963). It seems in the present work that the observed stimulation of lipase by PABA had been through synthesis of folic acid thus raising its level in the cells. It appeared from results that 50 ppm PABA are required to synhesize folic acid enough to stimulate lipase production. The folic acid optimal effect on lipase production was when 10 ppm of it were supplied to the medium. Requirement of 50 ppm of PABA for maximum stimulation of lipase and 10 ppm of folic acid would give evidence of the above claim that PABA worked throgh its contribution to folic acid synthesis. It is known that among the functions of folic acid is its use in the synthesis of purines and thymine (Harper,1967) which would imply its role in growth of microorganisms. The patterns mentioned above show similarities to the finding in some investigations (e.g. Peters & Nelson, 1951; Aflord et al., 1964 and Elwan et al., 1982) and differences to those in others (e.g. Alford and Purce, 1963 with *Ps. fragi* and Pelers & Nelson, 1948 with *M. lipolytica* and Elwan et al., 1982 with *P. lanosum*.

Indoles (particularly indole acetic acid IAA) gibberellic acid GA_3 are growth factors required for growth of plant cells. There are reports of their occurrence in and effect on certain processes in microorganisms (e.g. Fedorov and Savkina, Elwan and El-Naggar, 1969 & 1972). Recent work claimed that GA_3 induced *de novo* synthesis of ∝-amylase (Filner and Varner, 1967) and protease (Jacobsen and Varner, 1967) and probably other hydrolases which are released by the cells (Jacabsen and knox, 1970). The present work gives evidence of a probable effect of indole acetic, indole propionic, indole butyric acid and gibberellic acid on the synthesis of lipases by the investigated strain of *T. domerquii*.

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