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The effect of some growth conditions on the Production of lipases by Azotobacter chroococcum, Rhizobium sesbani and R.lupini, 282.

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Abstract: The present work was designed in order to study the effect of different carbon and nitrogen sources, vitamins and growth factors (Indoles and gibberellic acid) on lipase(s) production by the nitrogen fixing bacteria; A. chroococcum, R.sesbani & R.lupini, 282. Results showed that sucrose was the best carbon source for lipase(s) production at concentration of 0.5% for A.chroococcum & 0.1-0.2% for both R.sesbani & R. lupini 282. Potassium nitrate was the best nitrogen source (in the presence of yeast extract, 0.05%) for all strains. In addition, peptone & gelatin gave the highest lipase(s) yield for all the strains, whereas KNO_3 covering the range of 0.2-10 g/L (in the absence of yeast extract) caused remarkable reduction in lipases productivity as compared with the presence of yeast extract under the same production conditions. Addition of yeast extract to the production medium exerted a profound stimulatory effect which exceeded many times that effect recorded by any of the introduced individual vitamins. Gibberellic acid introduced at 350 p.p.m. was stimulatory to lipase(s), although inferior to yeast extract in case of A.chroococcum, similar to yeast extract in case of R.sesbani and twice that effect of yeast extract in case of R.lupini, 282 under the same production conditions.

INTRODUCTION

Although microbial lipase(s) in general received relatively high attention in our laboratory (Ammar, 1983; Ammar & McDaniel ; 1984 Ammar et al, 1984 & 1985 "a & b" ; Ammar et al, 1986 ; ElWan Et al, 1985 a & b, 1986 and 1986a & b). yet very little is Known about lipase from nitrogen fixing bacteria (ElGamal & ElSheikh, 1989; ElGamal & Rah 1989; Ammar et al , 1990).

In a previous work (Ammar et al,1990), an investigation concerning lipases from the nitrogen fixing bacteria (Azotobacter chroococcum Rhizobium sesbani & R.lupini, 282 was carried out. In the present work, lipase productivity from the same nitrogen fixing bacteria in relation to carbon & nitrogen sources, vitamins, indoles & gibberellic acid was undertaken.

MATERIALS AND METHODS

Organisms used: The same three lipolytic nitrogen fixing bacteria previously investigated for some factors affecting their ability to produce lipases (Ammar et al, 1990) were used. They were ; Azotobacte chroococcum,Rhizobium sesbani & R.lupini, 282.

Media used: A) Maintenance and growth medium: The basal medium of Vincent (1970) was modified to include the following constituents: 10 g sucrose ; 0.6 g KNO_3 ; 1 g KH_2PO_4; 0.25g $MgSO_4 7H_2O$; 0.1g $Cacl_2$ $6H_2O$; 10g Agar Agar; distilled water upto 1 L.

Production medium:- It contained the same components of the maintenance and growth medium with the addition of yeast extract at the level of 0.5 g/L and decreasing the concentration of sucrose down to 0.1% (W/V) as well as supplementing olive oil at the level of 2% (v/v). pH was adjusted at 7.0.

Lipase assay: The tributyrin-cup plate clearing zone(T.C.Z) of ElWan et al (1977) was applied for assaying lipolytic productivities.In this method : 0.2 % (v/v) of tributyrin was emulsified & supplemented with 2%(w/v) agar for solidification; pH was adjusted at 8.8 using Tris- Hcl buffer (hydroxymethyl amino methane-Hcl). After sterilization, equal amounts of assaying medium were dispensed

into plates. Cups were made in each plate using sterile cork borer. Astock solution of pancreatic lipase (Merck) was prepared. different concentrations of stock solution were prepared. 0.1 ml of each concentration was transferred into each cup & the clearing zones were determined after 6h incubation at 30°C. By plotting logs of enzyme concentrations (ug/ml) against mean diameters of clearing zones (in mm) a standard curve could be constructed from which the recorded enzyme concentrations (ug/ml) corresponding to the mean diameter of any clearing zone(mm) for each applied sample could be determined.

Some Factors affecting lipase(s) Production:

1- Effect of both carbon sources & Sucrose concentration:

A.chroococcum; R.sesbani and R.lupini 282 were allowed to grow in a sugar free production medium. Different sugars were added separately into the production medium at equimolecular amounts of the carbon found in 0.1% sucrose. In case of both starch and dextrine only 1 g/L. was added. In a further experiment the organisms were grown in sugar free production medium, supplemented with different levels of sucrose covering the range of 0.5-10 g/L. The pH was made at 7.0 & incubation was carried out at 30°C for 5 days. The bacterial filtrates of these strains were determined for their lipolytic activities by applying T.C.Z techniqu (ElWan et al,1977).

2- Effect of both nitrogen sources and KNO_3 concentration:

The three bacterial strains were grown in the basal production medium. Different nitrogen sources were added to the production medium at an equivalent nitrogen to that found in 0.06% KNO_3 in the basal medium. In addition, the same organisms were allowed to

grow in the production medium free from any nitrogen source & yeast extract but supplemented with KNO_3 at a series of concentrations ranging from 0.2-10 g/L. Other conditions were applied as mentioned before.

3) Effect of vitamins, indoles, gibberellic acid and different cencentrations of gibberellic acid:

The three organisms were grown in the basal production medium free from yeast extract. Various Vitamins, indoles and gibberelli acid were added to the production medium to replace the yeast extract at the concentration of 200 p.p.m. In addition, the same organisms were grown in the basal Production medium free from yeast extract but supplemented with GA_3 at a series of concentrations covering the range of 50-450 p.p.m to replace yeast extract pH was made at 7.0 & incubation was performed at 30°C for 5 days

Preparation of the cell free culture filtrates :

At the end of incubation period, the culture filtrate for each bacterial strain was centrifuged at 12,000 r.p.m. for 15 minutes a 4°C & the obtained supernatant was passed through a G-5 sintered glass funnel & then stored in a refrigerator after assaying its lipolytic activity according to Elwan et al (1977).

Inoculum size: The three bacterial strains were allowed to gr in the growth agar medium for 5 days at the end of which the bact growth was harvested for each strain & suspended into a sterile saline solution to obtain a stock bacterial suspension containing a definite number of cells about ($5x10^6$ cells/ml). Only one ml of stock bacterial suspension of each strain was transferred into 20 of the production medium dispensed into 100 ml conical flasks und sterile conditions.

Results & Discussion

There is a growing interest in microbial lipases in a number of old & new applications. Lipases containing detergents have been known since the introduction of enzyme detergents in 1914(Aunstrup, 1978), but the performance has not been satisfactory due to low activities of lipases. There is a renewed interest in lipases containing detergents because with the usual laundering process, fat stains are difficult to remove from some fabrics. Improved presoak preparations have been developed & satisfactory results were obtained by using lipase in combination with synthetic activators such as naphtholene sulfonates (Stewart et al ,1976). For the purpose of developing new detergent preparations it is beleived that introducing lipases from a relatively new virgin source e.g nitrogen fixing bacteria may solve problems in the future of laundering technology. Accordinglly, data in this work are complementary to that previously reported by Ammar et al (1990) which lay a great stress on the factors affecting lipase(s) from nitrogen fixing bacteria in general & obtaining a high yields of lipase(s) capable of attacking complex natural triglycerides as well as simple ones in particular.

Since Knowledge of Carbon nutrition is fundamental to an understanding of the physiology of bacteria, much valuable work has been done in this area , but the data required for confident generalization are still lacking & much of our knowledge is based on experiments which are open to criticism on the ground of method applied. This might be true since most of the available data are obtained from non nitrogen-fixing bacteria, but when it comes to the fact that such data are obtained from true, lipolytic N-fixers, the problem will be more interesting. Therefore,

there is a true need for more knowledge about the C-nutrition of lipolytic N-fixers. Thus, the effects of different C-sources on the lipolytic productivity of N-fixers under investigation were investigated.

Results in Table(1) showed that sucrose was the best carbon source for lipase(s) production by A.chroococcum ; R.sesbani and R.lupini,282 followed by glucose, starch, lactose, mannitol, maltose,D(+)Xylose, dextrine, D(+)mannose and L-arabinose for A.chroococcum,dextrine, mannitol, glucose, D(+)mannose, starch, lactose, L-arabinose, maltose and D(+)xylose for R.sesbani and starch, mannitol, D(+)mannose, maltose, L-arabinose, dextrine , lactose, glucose and D(+)xylose by R.lupini282, These results are in accordance with those reported by Ammar & McDaniel (1979 & 1984) & El-Hoseiny (1986) who found that sucrose was the best carbon source for lipases production. On the other hand, data in Table (1) show that starch has a moderate effect on the production of lipases by A.chroococcum & R.lupini 282. These results agree to a certain extent with those reported by ElWan et al, (1978 & 1986) who stated that addition of starch into the production medium stimulated lipases yield by Thermoactinomyces vulgaris & Penicillium chrysogenum. Data in Table (2) indicated that increasing sucrose concentration from (0.5-10 g/L) was accompanied by enhancement in lipases yield up to 0.5% (w/v) for A.chroococcum and 0.2% for both R.sesbani & R.lupini 282 beyond which an inhibition of lipases yield was recorded. It can be concluded from these results that the stimulation of lipases production by the addition of sucrose in the presence of olive oil could be explained on the bases that lipases produced belong to the inductive type.

Table (1) : Effect of different carbon sources equivalent to that of 0.1% sucrose on lipase(s) production by A.chroococcum; R.sesbani; and R.lupini 282

Carbon Sources	Lipase(s) Productivity (ug/ml). A.Chroococcum	R.sesbani	R.lupini 282
Lactose	156.250	39.062	156.250
Starch	312.500	78.125	312.50
Sucrose	1250.00	625.00	625.00
Dextrine	78.125	312.50	156.25
D(+)Xylose	156.250	78.90	19.50
Glucose	312.50	312.50	78.125
Mannitol	156.25	312.50	312.50
D(+)mannose	39.062	156.25	312.50
L-Arabinose	39.06	39.06	312.50
Maltose	156.25	39.06	312.50

Table (2): Effect of varying concentrations of sucrose on lipase(s) production by A.chroococcum; R.sesbani and R.lupini 282.

Sucrose conc. (g/L)	Lipase(s) Productivity (ug/ml). A.chroococcum	R.sesbani	R.lupini, 282
0.5	312.50	39.06	39.06
1.0	625.00	312.50	312.50
2.0	1250	312.50	312.50
5.0	2500.00	156.250	156.25
10.0	625.00	156.250	156.25

With regard to the effect of different amino acids on lipases production, results in Table (3) indicated that KNO_3 is superior to all the amino acids applied. It was followed by L-histidine, L-methionine, tryptophane, D.L-alanine(2500 ug/ml), L-glutamic aicd &

Table(3) Effect of supplying different amino acids at an equivalent amount of nitrogen to that of 0.06% KNO_3 on lipase(s) production by A.chroococcum; R.sesbani and R.lupini,282.

Nitrogen Sources	Lipase(s) Productivity (ug/ml). A.chroococcum	R.sesbani	R.lupini 282
KNO_3(control)	5000.00	2500.00	2500.00
L-Histidine	2500.00	156.250	2500.00
L-Asparagine	312.500	78.125	625.00
L-Methionine	2500.00	39.062	39.062
L-Glutamic acid	625.00	156.25	19.500
L-Valine	39.062	156.25	156.25
L-Arginine	625.000	312.50	312.50
Tryptophane	2500.00	156.25	312.50
D-L-Threonine	312.50	78.125	625.00
Glycine	78.125	156.25	39.06
D-L-Tyrosine	78.125	19.50	19.50
Isoleucine	78.125	1250.00	19.50
D-L-Alanine	2500.00	312.50	2500.00
L-Cystine	625.00	156.25	19.50

L-arginine & L-Cystine (625 ug/ml) & L-asparagine & D-L-threonine (312.50 ug/ml) ,whereas glycine D-L-tyrosine, isolucine and L-Valine were the least inducers for lipases production by A.chroococcum. In case of R.seshani, the remarkable effect of KNO_3 was followed by isoleucine, D.L.alanine,L-arginine, L-histidir

L-glutamic acid, L-valine, tryptophane, glycine, L-cystine; L-asparagine, D.L.threonine, L-methionine and D.L. tyrosine respectively. In case of R.lupini 282, Data in Table(3) showed that both KNO_3, L-histidine and D.L.alanine were the best nitrogen sources for the production of lipases, followed by L-asparagine, D.L. threonine, L-arginine, tryptophane, L-valine, followed by glycine, L-methionine, L-glutamic acid, D.L.tyrosine, isoleucine and L-cystine respectively. In view of the findings of other investigators, lipase production by Pseudomonas fragi(Nashif & Nelson, 1953) in glucose & citrate defined media was increased materially by supplementation with L.leucine or a combination of L.leucine, D.L-leucine & D.L. valine. However, it seems that amino acid which support growth & lipase production in the absence of glucose furnish C as well als Nrequired for cellular synthesis & enzyme production. However, Alford & Pierce(1961) observed that Pseudomonas fragi does not produce any lipase in a mineral glucose medium, but in supplementation with arginine, glutamic acid, aspartic acid & lysine does so in considerable amounts. Thus, it seems that the presence of amino acids in the growth medium is necessary for the production of lipase by Pseudomonas. However, P.fragi demands glucose for the production of lipase & in its absence no lipase is produced even in a medium containing the required minerals & amino acids (Alford & Pierce, 1963). On the other hand, Nadkarni (1971) reported that 0.2-0.4% of glutamic acid and 0.2-0.6% of tryptophane are suitable for maximum production of lipase.

Concerning the role of organic & inorganic nitrogen sources on lipases production by A.chroococcum, data in Table(4) indicated that gelatin was the best organic source followed by peptone in all cases. Whereas, the inorganic nitrogen sources did not fulfil the requirements for lipases induction by all strains. KNO_3 was the best under all conditions. These results are different from those reported by Ammar & McDaniel(1979 & 1984); Thiobodean & Nacy (1942) who found that some strains of B.stearothermophilus & Penicillum roquefortii preferred ammonium than nitrate ion for lipases production. Such differences could be attributed to difference in the organisms used. On the other hand, the fact that KNO_3 is the best inorganic N-source for lipases production by the N-fixers under investigation is similar to that recorded by Ammar et al (1984) with B.stearothermophilus, S.98 lipase. However, the utilization of NO_3^- rather than NH_4^+ may be interpreted by the fact that N-fixers in general- prefer NO_3^- rather than NH_4^+ & one may consider that N No_3^- is the excellent source of N for N- fixers. This might be supported also by the fact that the 3 N-fixers under investigation exhibited reasonable growth as well as lipase production in the basal production medium used at the begining of this work (Ammar et al., 1990). Since the basal production medium contained only NO_3^- as N-source & sucrose as C-source,this might suggest that the present N-fixers are self sufficient for all vitamins & can utilize nitrate & sulphate as a source of N & S respectively. Similar results were reported by cooney & Emerson(1965). Results in Table (5) indicated that low lipases production occured in presence of KNO_3 at the levels of (0.2-10g/L) when the production medium was free from yeast

extract. Addition of yeast extract into the production medium without KNO_3 gave high lipases production by all the bacterial strains as shown in Table(5) . These results (Table 5) reflect the importance of yeast extract for both lipase(s) production as well as bacterial growth. No doubt that yeast extract plays an important role in the growth & subsequently lipase biosynthesis by most lipases producers (Arima et al , 1972; Elwan et al,1977 and 1978a &b ; . Ammar & McDaniel ; 1979 & 1984; Ammar, 1983). Therefore, a trial was made to investigate the role of vitamins,indoles & gibberellic acid on lipases production. Results in Table(6) showed that individual vitamins, indoles and GA_3 were inferior to yeast extract. However, Gibberellic acid was similar to individual vitamins as well as to the growth factors tested. Experiments on the effect of different concentrations of gibberellic acid on lipases production by the three bacterial strains (Table 7) show that optimal concentration for both A.chroococcum & R.lupini 282 was 350 p.p.m. and 300-350 p.p.m. for R.sesbani . These results agree- to a certain extent with that mentioned by El Gamal & Rahal(1989) who found that gibberellic acid at a certain concentrations (10 and/or 20 p.p.m.) stimulated lipases productivity by some strains of R.japonicum.

Table(4) : Effect of organic & inorganic nitrogen sources on lipase(s) production by A.chroococcum ; R.sesbani; and R.lupini,282.

Nitorgen Source	Lipase(s) Productivity (ug/ml). A.chroococcum	R.sesbani	R.lupini 282
KNO_3(control)	5000.00	2500.00	2500.00
Peptone.	156.25	1250.00	625.00
Urea.	156.25	156.25	156.25
Gelatin	312.50	2500.00	2500.00
Sodium nitrite	39.06	9.750	39.06
Ammonium molybdate	156.25	78.125	39.06
Ammonium nitrate	39.06	312.500	78.125

Table(5) : Effect of potassium nitrate (in the absence of yeast extract) on lipase(s) producation by A.chroococcum; R.sesbani and R.lupini, 282

KNO_3conc.(g /L.)	Lipase(s) Productivity (ug/ml) A.chroococcum	R.sesbani	R.lupini 282
0.0(Control)	0.600	0.600	0.037
0.2	4.87	2.438	0.600
0.5	9.75	4.87	2.438
1.0	19.50	9.75	4.87
2.0	39.06	19.50	4.87
3.0	19.50	4.87	2.438
5.0	19.50	4.87	2.438
10.0	19.50	4.87	2.438
KNO_3(0.1%)+yeast extract(0.05%)	2500.00	625.00	312.50
Yeast extract(0.05%) +NO KNO_3	2500.00	312.50	312.50

Table(6) Effect of different vitamins, Indoles and Gibberellic acid in absence of yeast extract on lipase(s) production by A.chroococcum; R.sesbani an R.lupini, 282

Substance	Lipase(s) Productivity (ug/ml). A.chroococcum	R.sesbani	R.lupini 282
Ascorbic acid	156.250	156.250	39.062
Riboflavin	312.50	39.062	39.062
Folic acid	156.250	39.062	78.125
Thiamine-Hcl.	312.500	39.062	78.125
Inositol	312.500	78.125	156.250
Nicotinic acid	312.500	156.25	156.25
Panthothenic acid	312.500	78.125	39.062
Pyridoxine-Hcl	156.250	39.062	78.125
Biotin	312.500	156.250	156.250
Indole propionic acid	156.250	39.062	78.125
Indole butyric acid	156.25	78.125	156.250
Gibberellic acid	156.250	156.250	156.250
Yeast extract	1250.00	625.00	312.50
(Both Vitamin & Yeast extract are absent)	9.75	4.875	2.438

Table(7) Effect of different concentrations of gibberellic aicd in absence of yeast extract on lipase(s) production by A.chroococcum; R. sesbani and R.lupini 282.

Gibberellic acid conc.(p.p.m.)	Lipase(s) Productivity (ug/ml). A.chroococcum	R.sesbani	R.lupini 282
0	9.75	4.875	2.438
50	78.125	39.062	39.062
100	78.125	78.125	78.125
150	78.125	78.125	78.125
200	156.250	156.250	156.250
250	156.250	156.250	156.250
300	312.50	625.00	312.50
350	625.000	625.00	625.00
400	312.50	312.50	312.50
450	312.50	312.50	312.50
Yeast extract	1250	625	312.50

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