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PRODUCTION OF ALKALINE PENICILLIN ACYLASE BY THE HALOALKALIPHILIC BACILLUS WN-14 SP-NOV-

By

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ABSTRACT

Bacillus No. WN - 14, a haloalkaliphilic bacterium, isolated from the Salt marshes of Wadi El - Natroun, Egypt, produced alkaline penicillin acylase enzme in alkaline medium. Physiological and biochemical charateristics proved to be a new haloalkaliphilic Bacillus species. Bacillus WN - 14 produced high yield of extracelluar alkaline penicillin acylase enzyme at 40 C, pH 10.0 in 0.2 M Borax - NaOH buffer, in the presence of starch (1.0 %), potassium phenyl acetate (25.0 %), NaCl (12 %), yeast extract (0.3 %) and phenyl alanine (0.6 %). The crude enzyme extract showed maximum activity at pH 10.0 and the product (6 - APA) was stable up to pH 11.

INTRODUCTION

The commercial importance of 6 - aminopenicillanic acid (6 - APA) for the production of semisynthetic penicillins has accelerated the development of penicillin acylase research and application, especially since the economy of the chemical route to penicillin G hydrolysis became critical because of rising energy costs (Schomer et al., 1984). The distribution of penicillin acylase in bacteria and fungi have been described in some recent reviews (Lowe et al., 1981; Vandamme, 1981, and Vandamme and Voets, 1974).

Penicillin G acylase (EC 3. 5. 1. 11: penicillin amidohydrolase) have been often reported to be an intracellular enzyme produced by some bacterial, actinomycete and fungal species with the exception of *B-megaterium* ATCC 14945 which was the only report on the production the enzyme extracellularly (Chiang and Bennett, 1967 and Vandamme, 1977).

Recently B - lactamases were reported to be produced by alkaliphilic bacteria (Kobayashi et al., 1986 and Kato et al., 1985). However, no report, as far as we know, has contributed on the production of extracellular alkaline penicillin G acylase. The present investigation reports the finding of the production of extracellular alkaline penicillin G acylase from the haloalkaliphilic bacterium *Bacillus* No WN - 14.

MATERIALS AND METHODS

Chemicals :

Potassium benzyl penicillin (Bp) was obtained from "Phone - polenc"; p-dimethyl aminobenzaldehyde (p-DAB) from "Carlo Erba"; 6-aminopenicillinic acid (6-APA) from "Rhom Pharma GMBH WELTERSTADT; TLC plastic sheets, Silica gel (Merck).

Media:

1- Potassium phenyl acetate - peptone - yeast extract medium (KPY) was used for the isolation and maintenance. It was formed of GPY medium supplemented with 10% NaCl according to Ghanem et al., (1990) modified by the elimination of glucose and addition of 0.15 % potassium phenyl acetate. PH of the medium was adjusted to 10.4 using 1 % Na_2Co_3.

2- Penicillin potassium phenyl acetate -peptone - yeast extract PKPY medium supplemented with 0.5 % potassium penicillin G in addition to potassium phenyl acetate was used for enzyme production, PH of the medium was adjusted to 9.6 using 0.6 % Na_2Co_3.

Characterization and identification:

Microbiological properties were investigated according to the methods described in Manual of methods for General Bacteriology (1984), Bergey's Manual of Systematic Bacteriology (1986) and Fritze et al., (1990).

Preparation of penicillin acylase :

Bacillus WN-14 was grown aerobically on KPY medium with shaking (new Brunswick shaker incubator) for 18 h. at 40 C. Cells were removed by centrifugation at 6000 X g for 10 min and the supernatant fluid was examined as a crude enzyme solution .

Confirmation of stability of penicillin G at various pHS:

Penicillin G was dissolved at PH ranges starting from 7.5 to 11, then assayed biologically against *Staphylococcus aureus* ATCC 29737. Results were expressed as diameter of the inhibition zone.

Confirmation of the stability of 6-APA at various pHS:

Same test as above with the use of *Serratia marcescens* ATCC 27117 as the test organism.

Confirmation of duration of stability of penicillin and 6 - APA at room temperature:

The effect of preservation period of both penicillin G and 6 - APA at high pH was investigated using the same test organisms mentioned above.

Qualitative detection of alkaline penicillin G acylase activity:

An enzyme solution 0.5 - 1.0 ml was incubated with 0.5 % penicillin G dissolved in 0.2 M phosphate buffer at pH 8.0 and incubated at 37 C for 30 min. Penicillin acylase activity was demonstrated by the detection of its end product: 6 - APA. Thin layer sillica gel plates (0.1 mm) were mounted with 0.1 ml crude enzyme solution, run using isobutanol: methylene

chloride: formic acid: H_2O (7: 1: 1: 1), and developed with Iodine - starch solution after treatment with ammonia fumes (Thomas, 1961). Penicillin G, 6 - APA and uninoculated broth were also mounted as reference and negative control.

Quantitative determination of enzyme activity:

Penicillin G acylase activity was measured in the crude extract according to the method of Bomstein and Evans (1965). Penicillin G was used as the working substrate as mentioned above. The reaction was based on the reaction of the free amino group of 6 - APA with the aldehyde group of p - dimethyl aminobezaldehyde (p - DAB) to from a colored Schiff's base. The intensity of the yellow color developed within 15 min was measured at 415 nm. The penicillin G acylase activity in the crude extract was calculated from the standard curve established by treating different concentrations (10 - 100 ug/ml) of 6 - APA by the above mentioned procedure. One unit of enzymatic activity is defined as the amount of enzyme required to produce 1 umol of 6 - APA per min under conditions described above.

Factors affecting alkaline penicillin acylase production:

The following factors were investigated: Effect of different buffers; different NaCl concentrations; different potassium phenyl acetate concentrations; different carbon sources; different nitrogen sources; different temperatures and the effect of adding penicillin G to the KPY medium.

Effect of different pHs on the activity of the crude enzyme:

Crude enzyme extracts were assayed with penicillin dissolved at different pH ranges from 4.5 to 12. Activity was measured by the p - DAB assay method mentioned above.

RESULTS AND DISCUSSION

Characters of *Bacillus* No. WN - 14: Many isolated alkaliphilic strains were screened for the production of alkaline penicillin acylase. Activity was qualitatively determined using thin layer chromatography on silica gel plates. All strains were previously isolated by Ghanem et al., (1990). Only one strain No WN - 14 showed alkaline penicillin acylase activity in the culture fluid. It was subjected to the following identification characters: Strain WN - 14 is aerobic, sporeforming, gram positive, motile, rod shaped bacterium, has peritrichous flagella. It is clear that the bacterium should belong to the genus *Bacillus*. The characteristic point of the bacterium was that the growth was very good in alkaline and saline media, and the optimal pH for growth was about 10.5 and optimal NaCl concentration was 10 %. No growth was detected in neutral media, such as nutrient broth. Table (1) summarizes the morphological and cultural characteristics of the strain WN-14. Taxonomic position of this strain will be reported elsewhere together with other Bacilli that grow preferentially or obligatorily in alkaline media and produce alkaline enzymes.

Culture conditions for the production of an alkaline penicillin acylase:

Results recorded in figure 1 (A to D) showed that the best cultural conditions for the production of alkaline penicillin acylase were as follows: 0.25 %, potassium phenyl acetate; 12 % NaCl; pH 10.0; 40 C; and 20 h. incubation period. On the other hand, the best nutritional sources were represented as starch, 1 %; phenyl alanine 0.6 %, and yeast extract, 0.3 % (Table, 2 and Fig. 2). Borax - NaOH buffer at pH 10.0 was the best buffer for the production of alkaline penicillin acylase. The addition of penicillin G as an inducer to the KPY medium had no effect on the productivity of the enzyme.

Detection of stability of penicillin and 6 - APA at various pHs:

Investigation of the stability of penicillin and 6 - APA at various buffers of different pHs was investigated biologically against *S. aureus* ATCC 29737 and *Serratia marcescens* ATCC 27117. Results (Fig. 3) showed that penicillin resisted pH as high as 10.0 and 6 - APA resisted pH as high as 11.0. Growth of *S. aureus* ATCC 29737, a sensitive test organism of penicillin G, showed gradual resistance to penicillin G after pH 10.0, indicating that pH 10.0 is a critical pH after which penicillin will be unstable. On the other hand *Serratia marcescens* ATCC 27117 showed also gradual resistance to 6 - APA above pH 11.0 indicating the instability of 6 - APA above this limit of pH.

Effect of preservation period on 6 - APA and penicillin G at the maximum pH:

Assay of penicillin G and 6 - APA after different preservation periods at their maximum pHs was performed against *S. aureus* ATCC 29737 and *S. marcescens* ATCC 27117 respectively. Results (Fig. 4) showed that, penicillin G was stable for a period of 3 days at pH 10.0 then gradual loss of stability occurred to reach maximum loss after 10 days. On the other hand, 6-APA was stable for 8 days under the specified conditions followed by slow loss rate of stability.

Effect of pH on the activity of the crude enzyme:

Results (Fig. 5) showed that crude alkaline penicillin acylase begins its activity at pH 8.4 and reaches maximum activity at pH 10.0 then activity decreased gradually with increased pH.

A large number of microorganisms have been found to produce penicillin acylase (Backer, 1992). Production pH and temperature ranged between pH 4.5 to 9.0 at 28 to 50 C. Only two strains were reported to produce penicillin acylase under pH 9 (Betina, 1983). The first one was *Micrococcus roseus* ATCC 516 which produced entirely intracellular enzyme at maximum pH 9 at 35 C using benzylpenicillin as substrate. The second was *Streptomyces lavendulae* which was reported to produce the enzyme in the culture filtrate at pH 8 - 9 and 40 - 50 C using phenoxymethylpenicillin as a substrate.

Numerous applications for enzymes or pharmacologically active chemicals that are stable at high pH. However, only B-lactamase and

pencilinase enzymes were reported as enzymes producted by alkaliphilic bacteria (Krulwich and Guffanti, 1989). The present paper has reported the production of extracellular alkaline penicillin acylase stable at pH up to 11.0 at 40 C, the end product also showed stability at pH 11 for a long period at room temperature and maximum enzyme activity was attained at pH 10.0.

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Table 1: Morphological, cultural and biochemical characteristics of strain No. WN - 14.

1- Morphological characteristics:		
From	Rods	
Size	0.5 - 0.8 u × 3.5 - 4.6 u	
Motility	motile	
Gram stain	positive	
Sporangia	slightly swollen	
Spores	0.9 - 1 u to 1.2 - 1.5 u; oval	
2- Cultural characteristics:		
	Growth at	
	pH 7	pH 10.5
Nutrient broth (NB)	-	+
Glucose NB	-	+
GPY	-	+
PPY	-	+
Glucose asparagine agar	-	+
Anaerobic growth	-	+
GPY + 10 % NaCl	-	+
3- Biochemical characteristics:		
Hydrolysis of gelatine and case in	Positive	
Hydrolysis of starch	Positive	
Utilization of citrate	utilized	
Reduction of nitrate	reduced	
Voges - Proskauer	Positive	
4- pH and temperature:		
pH in GPY medium	pH 8.5 - 11.8	
pH in KPY medium	pH 8.5 - 11.8	
Temperature	25 - 50	

Table 2: Effect of different nutritional sources on Penicillin acylase production by strain WN - 14.

1- Cabon sources:	Penicillin acylase activity (unit/ml)
D - glucose	25.5
D - galactose	19.9
D - mannose	20.8
D - fructose	18.6
D - mannitol	20.6
Sucrose	24
Lactose	20
Maltose	20
Raffinose	5
Trehalose	21
Mellibiose	20
Erythritol	20.6
Inositol	not detected
Inulin	20
Starch	43.4
2- Nitrogen sources:	
Sodium nitrate	19.5
Amm. sulfate	21
Urea	18.8
Peptone	not detected
Valine	18.3
Serine	20
Methionine	22
Tryptophane	24
Phenyl alanine	28
Proline	22
Arginine	19
Alanine	23
3- Vitamins:	
Yeast extract	31.5
Nicotinic acid	not detected
Thiamine	15.4
Riboflavine	17.5
Biotin	not detected
Pyridoxine	16.5
Folic acid	not detected
Sodium pantothenate	not detected

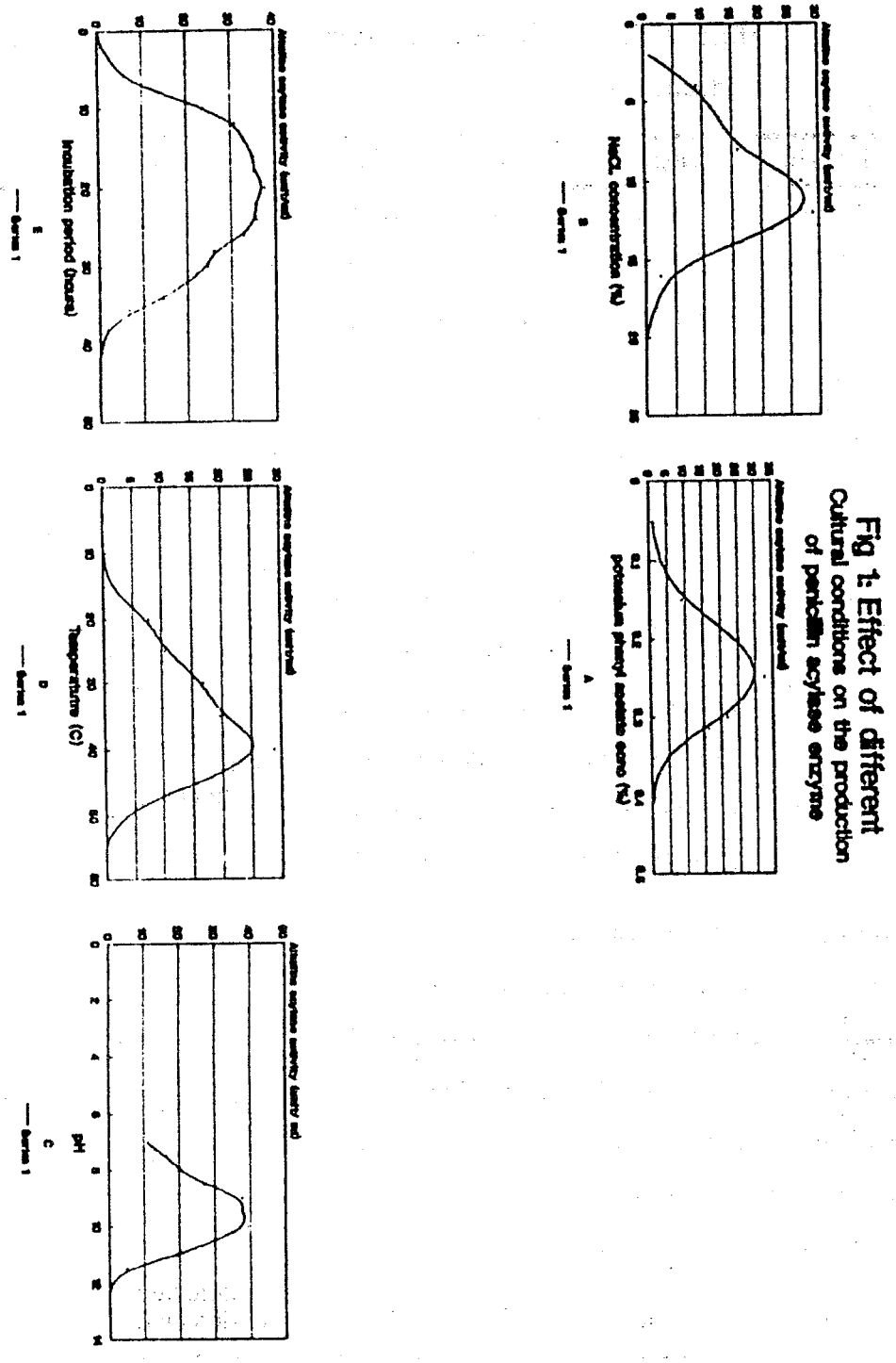

Fig 1: Effect of different Cultural conditions on the production of penicillin acylase enzyme

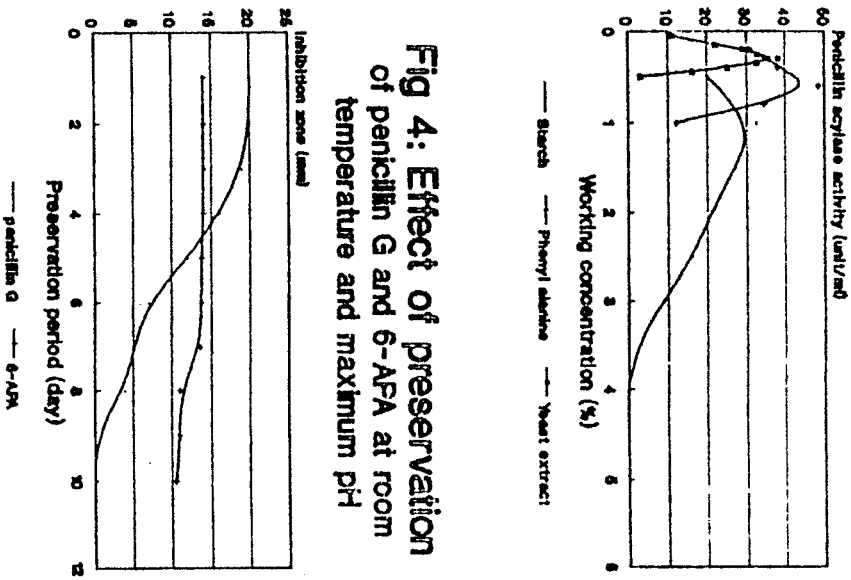

Fig 2: Effect of different concentrations of the best nutritional source.

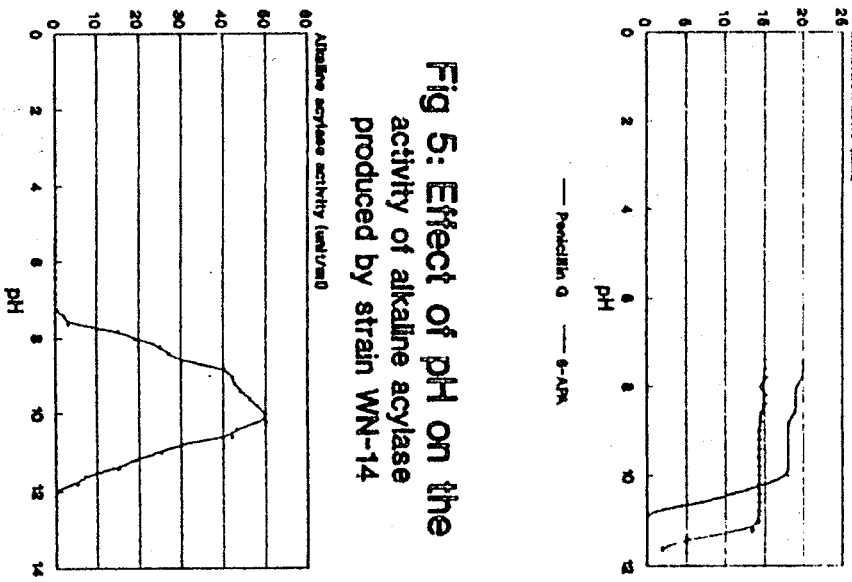

Fig 3: Effect of pH on the stability of penicillin G and 6-APA using *S. aureus and S. marcescens*

Fig 4: Effect of preservation of penicillin G and 6-APA at room temperature and maximum pH

Fig 5: Effect of pH on the activity of alkaline acylase produced by strain WN-14