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ANTISTAPHYLOCOCCAL METABOLITE FROM *AUREOBASIDIUM PULLULANS*: PRODUCTION AND CHARACTERIZATION

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Aureobasidium pullulans **(NI.3) isolated from the leaves of *Dracaena reflexa variegata* produced intracellular antimicrobial metabolite the yield of which was 700-800 U from about 0.7-0.85 g of dry biomass. The antistaphyloccocal metabolite showed strong activity against different *Staphylococcus spp.* The MICs ranged from 1.25 to 3.6 U/ml. The metabolite was only moderately sensitive to temperature. After storage at 40°C and 70°C for one hour, it lost only 20% and 60% of its activity respectively. However, it was completely inactivated upon exposure to 121°C for 20 min. The antistaphyloccocal metabolite was insensitive to various protein-denaturing detergents and enzymes like trypsin, proteinase K, lipase and lysozyme. The activity was fairly stable over a wide range of pH (5.7 – 8). When *S. aureus* was grown in the medium in presence of antimicrobial metabolite (10 U/ml), the number of CFU progressively declined, with most of the cells loosing their viability after nine hours exposure. A slower killing of the *S. aureus* was noted when cells were kept in buffer containing antimicrobial metabolite (5 U/ml). Antimicrobial metabolite induced efflux of potassium ions from cells of Staphylococcus indicating that the mode of action is by formation of pores or channels in the cytoplasmic membrane.**

Key words: *Aureobasidium*, Antistaphylococcal activity, Potassium efflux

INTRODUCTION

Of the six thousand and more microbial metabolites described to date, four thousand are antibiotics, 1% of which has been used clinically (1). More than three thousand antibiotics have been isolated from *Actinomycetes* and a few from fungi (2). The large number of other microorganisms are yet unexplored for their potential to produce antibiotic. Screening of unusual fungi and other microorganisms not yet explored from the natural sources around the world might be productive in yielding new bioactive metabolites including antimicrobial metabolites.

Aureobasidium pullulans, a fungus, has not been adequately investigated for its antimicrobial potentials. Only few studies regarding its ability to produce antimicrobial compound are reported (3-4), however, detail investigation is necessary. In this paper, we report on production, characterization and mode of action of intracellular antimicrobial metabolite from indigenous strain of *A. pullulans* NI. 3.

MATERIALS AND METHOD

Organisms and their cultivation

Aureobasidium pullulans NI.3 culture was maintained on Saboraud dextrose agar, *Staphylococcus aureus* reference strain for assay, *Staphylococcus* MGHM 1 (Mahatma Gandhi Hospital, Mumbai), *Staphylococcus* MGHM 2, *Staphylococcus* MGHM 3, *Staphylococcus* MGHM 4, *S. aureus* NCIM 2079 (National Collection of Industrial Micro-organisms, National Chemical Laboratory, Pune, India.) and *S. epidermidis* MTCC 435 (Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India) were maintained on nutrient agar slants and sub cultured every month and stored at 4-10°C until studied.

Isolation of A. pullulans from leaves of Dracaena reflexa variegata

A. pullulans NI.3 strain was isolated from *Dracaena reflexa variegata* leaves by using procedure described by Pollock *et al* (5), identified and characterized by procedure of Barnett *et al* and Takeo and Hoog (6-7).

Fermentation

Inoculum was prepared by transferring a loopful of cells from slant of *A. pullulans* in 10 ml of Saboraud Dextrose Broth (SDB), which was incubated for 24 hr at 28⁰C on shaker (120 rpm). One ml of inoculum was added to 100 ml of medium, containing (g/100 ml) glucose 2.0, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 0.15, $MgSO_4$ 0.5, $CaCl_2$ 0.01, $FeCl_3$ and $ZnSO_4$ 5 µg/ml in 500 ml flask and incubated at 28⁰C on shaker. After two days of incubation, the medium was replenished with 20 ml of the same medium containing peptone 5%, and culture growth was continued for further four days.

The fermented broth was centrifuged at 5000 rpm for 20 minutes. Biomass was suspended in equal volume of ethanol and grounded in mortar and pestle with coarse silica for 15 – 20 minutes and centrifuged at 5000 rpm. The ethanol extract was evaporated to dryness and the residue was dried under the stream of nitrogen gas. During the fermentation, pH and biomass were measured.

Assay of antimicrobial activity

The dry residue obtained at the end of extraction was dissolved in 2 ml of 95% ethanol, and was loaded to paper disc as follows; 10 discs (Whatman paper No.1, disc diameter 5 mm) were soaked in 100 µL of ethanolic extract and kept at room temperature overnight. The discs were placed on nutrient agar which has been previously inoculated with target culture bacteria. Plates were incubated for 24-48 hours at 37⁰C and the zone diameters of inhibition were measured. Discs similarly prepared using 95% ethanol alone served as control. One unit of antibiotic was the amount of antibiotic which gave 5 mm zone diameter of inhibition against *S. aureus*.

Determination of MICs of antimicrobial metabolite from A. pullulans NI. 3

The minimum inhibitory concentration of target organisms was determined by the Kirby-Bauer double dilution method (8).

Measurement of potassium efflux

S. aureus cells were suspended in 25 ml of 2.5 mM Na-HEPES buffer, pH 7.2 containing 6.24 units/mL of antimicrobial metabolite from *A. pullulans* NI.3 and kept for two hours at room temperature. 5 ml samples were withdrawn at different time intervals, centrifuged at 12, 000 rpm for 10 minutes and the supernatants were collected and analyzed for K^+ using flame photometer. *S. aureus* cells suspended in 25 ml of Na-HEPES buffer alone were used as control. Potassium released after heat treatment of cultures at 100⁰C for 10 minutes was used as reference.

Characterization of antimicrobial metabolite from A. pullulans NI.3

The effects of temperature, pH, detergents and enzymes on antimicrobial metabolite were determined.

RESULTS

Production of antimicrobial metabolite by A. pullulans NI.3

The production of intracellular antimicrobial metabolite started after 24 hours and continued almost at a constant rate for 5 more days. During the same period, biomass as measured in terms of dry weight also kept increasing. The maximum amount of antimicrobial activity extracted from biomass was 700-800 U from about 0.7-0.85 g of dry biomass (Fig.1).

The antimicrobial metabolite of *A. pullulans* NI.3, determined by agar disk diffusion assay, is summarized in Table 1. The extract of *A. pullulans* NI.3 produced well-defined inhibition zone diameter of 14-23 mm against *Staphylococcus spp.* Absence of inhibition zones with control ruled out possibility of inhibition due to residual ethanol.

Effect of temperature and pH on antimicrobial activity of metabolite

The effects of temperature and pH on antimicrobial activity of metabolite against *Staphylococcus spp* was analyzed in term of residual activity (Fig. 2). When the metabolite was exposed to various temperatures, the loss of activity was gradual; at 50ºC more than 70% of activity was still retained. After exposure at 70ºC for one hour, there was only 60% of loss of activity as tested against *S. aureus*. However, the activity of the extract against *S. aureus* was lost completely after autoclaving. The antimicrobial metabolite was fairly stable for 34 days at room temperature showing loss of 46% of its activity as tested against *S. aureus*. It retained 84% of antimicrobial activity even after eight days of incubation at room temperature (Data not shown). The antimicrobial substance was very stable over a wide range of pH 5.7 – 8.0 (Fig. 2).

Effect of enzymes and protein-denaturing detergents on antimicrobial metabolite

Antimicrobial metabolite was treated with a variety of detergents and enzymes (Table 2). No loss of antimicrobial activity of the metabolite was observed upon treatment with any of the detergents except bile salt. In case of treatment with bile salt, zone diameter decreased from 24 to 19 mm. Upon treatment with proteinase K, trypsin, lysozyme and lipase, the activity of antimicrobial substance against *S. aureus* remained largely unaffected (Table 3).

Effect of antimicrobial metabolite from A. pullulans on growth and survival of S. aureus

Significant inhibitory effects were observed when antistaphylococcal metabolite (10 U/ml) was added to the growth medium (Fig. 3). After 3 hours of contact with the metabolite, more than 1.5 $\log_{10}$ CFU decrease was observed. A 6 hours incubation with antistaphylococcal metabolite led to killing of 8 $\log_{10}$ CFU decrease. In the controls, plain LB as well as LB containing 125 µL ethanol, the initial cell number increased by at least 1 $\log_{10}$ CFU. Similarly, when *S. aureus* cells was suspended in phosphate buffer containing antimicrobial metabolite (5U/ml), the viable cell count of *S aureus* decreased by 1.5 $\log_{10}$ CFU (Fig. 4).

Effect of reversal agents on activity of antimicrobial metabolite from A. pullulans NI.3

In order to determine whether antistaphylococcal metabolite from *A. pullulans* NI.3 inhibit any biosynthetic pathway, the metabolite was mixed with reversal agents and residual activity was determined. Inhibition of *S. aureus* was not reversed by any of the tested reversal agents (Table 4).

Determination of K^+ efflux from Staphylococcal cells in presence of antistaphylococcal metabolite from A. pullulans NI.3

In order to further understand the mechanism of action of antistaphylococcal metabolite from *A. pullulans* NI.3, we investigated the kinetics of potassium efflux from the cells (Fig. 5). Following incubation of cells with antistaphylococcal metabolite, the extracellular potassium concentration was significantly increased. The concentration of K^+ outside cells, treated with the metabolite for 10 minutes was 5 ppm and increased up to 6 ppm in 30 minutes. The largest amount of potassium released within 2 hours of incubation was 7 ppm. Extracellular levels of K^+ were negligible from *S aureus* suspended in buffer containing alcohol.

Table 1: Inhibition of *Staphylococcus spp* by antimicrobial metabolite from *A. pullulans* NI.3

Target organisms	Inhibition zone diameter † (mm)	MICs (U/ml)
Staphylococcus sp MGM 1	14	2.8
Staphylococcus sp MGM 2	18	3.6
Staphylococcus sp MGM 3	18	3.6
Staphylococcus sp MGM 4	15	3
S. aureus	24	1.25
S. aureus NCIM 2079	23	3
S. epidermidis MTCC 435	22	1.56
Control *	0	0

MIC values were determined by Kirby-Bauer double dilution method

* Disc containing 10 µl ethanol was kept as control; †each value represents the average of three measurements

Table 2: Effect of detergents on antistaphylococcal metabolite from *A. pullulans* NI.3

Detergents	Inhibition zone diameter † (mm)	
	No metabolite ª	Metabolite
Tween 80	0.0	21
Tween 20	0.0	21
Triton X 100	0.0	21
SDS (0.01 g/ ml)	0.0	20
Cetrimide (0.01 g/ ml)	13	23
Bile salt (0.01 g/ ml)	0.0	19
Toluene	10	22
No detergents	0.0	24

Aliquots (200 µL) containing 96 units of antistaphylococcal metabolite were mixed with 20 µL of detergents and incubated for three hours at room temperature. The antistaphylococcal activity was then checked by using discs as described in materials and methods

*Control: 200 µL of antimicrobial metabolite mixed with 20 µL of ethanol.

ªDetergent was mixed with distilled water (200 µL)

† Each value represents the average of three measurements.

Table 3: Effect of enzymes on antistaphylococcal metabolite from *A. pullulans* NI. 3

Test	Inhibition zone diameter † (mm)
Trypsin + NI. 3 extract	12
Proteinase K+ NI. 3 extract	11
Lipase + NI. 3 extract	13
Lysozyme + NI. 3 extract	13
* Diluted NI. 3 (Control)	11

100 µL of antistaphylococcal metabolite containing (44 units) was added to 100 µl of enzyme solution (1 mg / ml) incubated at room temperature for three hours, 100 µl of the mixture was added to 10 sterile discs. Using these discs the antistaphylococcal activity was determined

* Antimicrobial metabolite was diluted to match with dilution caused by addition of enzyme

† Each value represents the average of three measurements.

Table 4: Reversal of antistaphylococcal activity of metabolite from *A. pullulans* NI. 3

Reversal agent			*A. pullulan* extract	Inhibition
	µL/well	µg /well	µL	zone dia † (mm)
Casamino-acids*	B 40	4	40	26
	B 40	4	60	37
Riboflavin	A 20	0.2	20	27
	B 40	0.4	60	40
Nicotinic acid	A 20	0.2	20	27
	b 40	0.4	60	38
Thiamine	a 20	0.2	20	26
	b 40	0.4	60	39
Adenine	a 20	0.2	20	28
	b 40	0.4	60	40
Cytosine	a 20	0.2	20	26
	b 40	0.4	60	39
Guanine	A 20	0.2	20	26
	B 40	0.4	60	38
Uracil	A 20	0.2	20	26
	B 40	0.4	60	39
Thymine	A 20	0.2	20	27
	B 40	0.4	60	40
H_2O	A 20	--	20	28

100 µl of *S. aureus* (OD $_{660}$ 0.7) each was spread inoculated on NA. The plate was incubated at 37°C. Concentration of stock solution for casamino- acids was 100µg/ ml and for all other reversal agents was 10 µg/ ml. a = 10mm diameter well; b= 5 mm diameter well; †each value represents the average of three measurements.

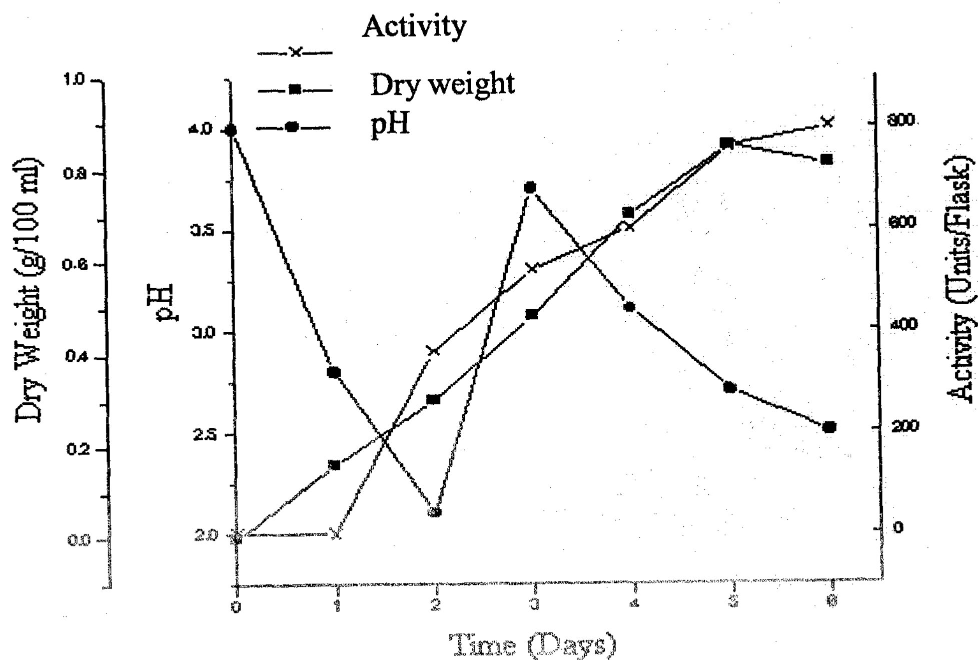

Fig. 1: Time course of production of antimicrobial metabolite *A. pullulans* NI.3
Production of antistaphylococcal metabolite was studied in shake flasks, incubated at 120 rpm on a shaker, at 30°C for six days. Samples were removed periodically and growth, pH as well as metabolite production were determined.

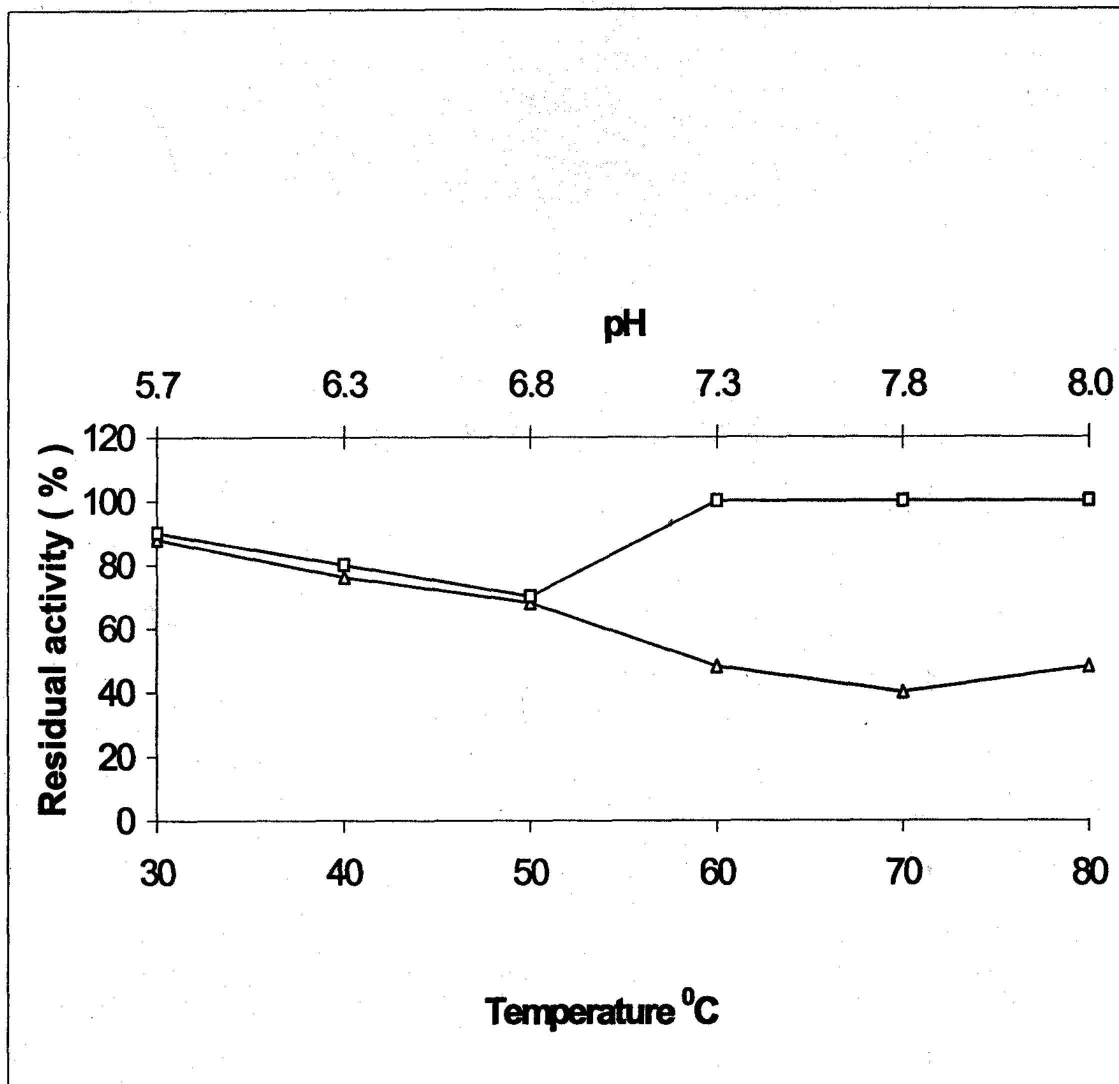

Fig. 2: Effect of temperature and pH on antistaphylococcal metabolite from *A. pullulans* NI.3
Aliquots (200 µl) of metabolite containing (88 units) were kept in water bath at different temperatures for one hour and residual antimicrobial activity for each aliquot was measured. Similarly, 200 µl (88 U) of antistaphylococcal metabolite was mixed with 200 µl of phosphate buffer of different pH and residual antimicrobial activity for each aliquot was measured.
□ = pH, Δ = Temperature

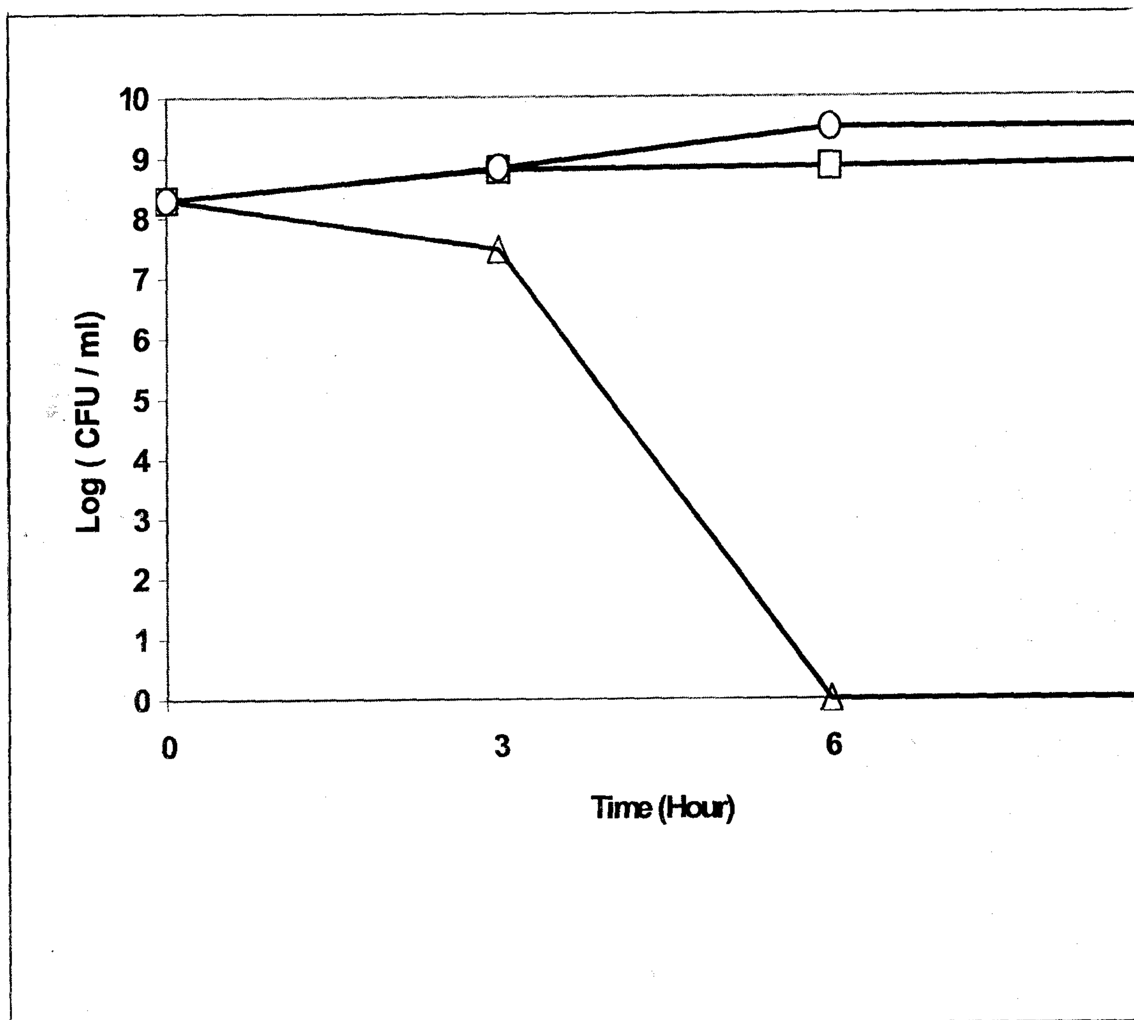

Fig. 3: Effect of antistaphylococcal metabolite from *A. pullulans* NI. 3 on growth of *S. aureus*
S. aureus cells were inoculated to 10 ml of LB (○), LB containing 125 µl ethanol (□) and to LB containing 10 U/ml of antimicrobial metabolite (Δ) and incubated for the indicated period at 37°C on shaker at 120 rpm. Growth was measured in terms of total viable counts (TVC).

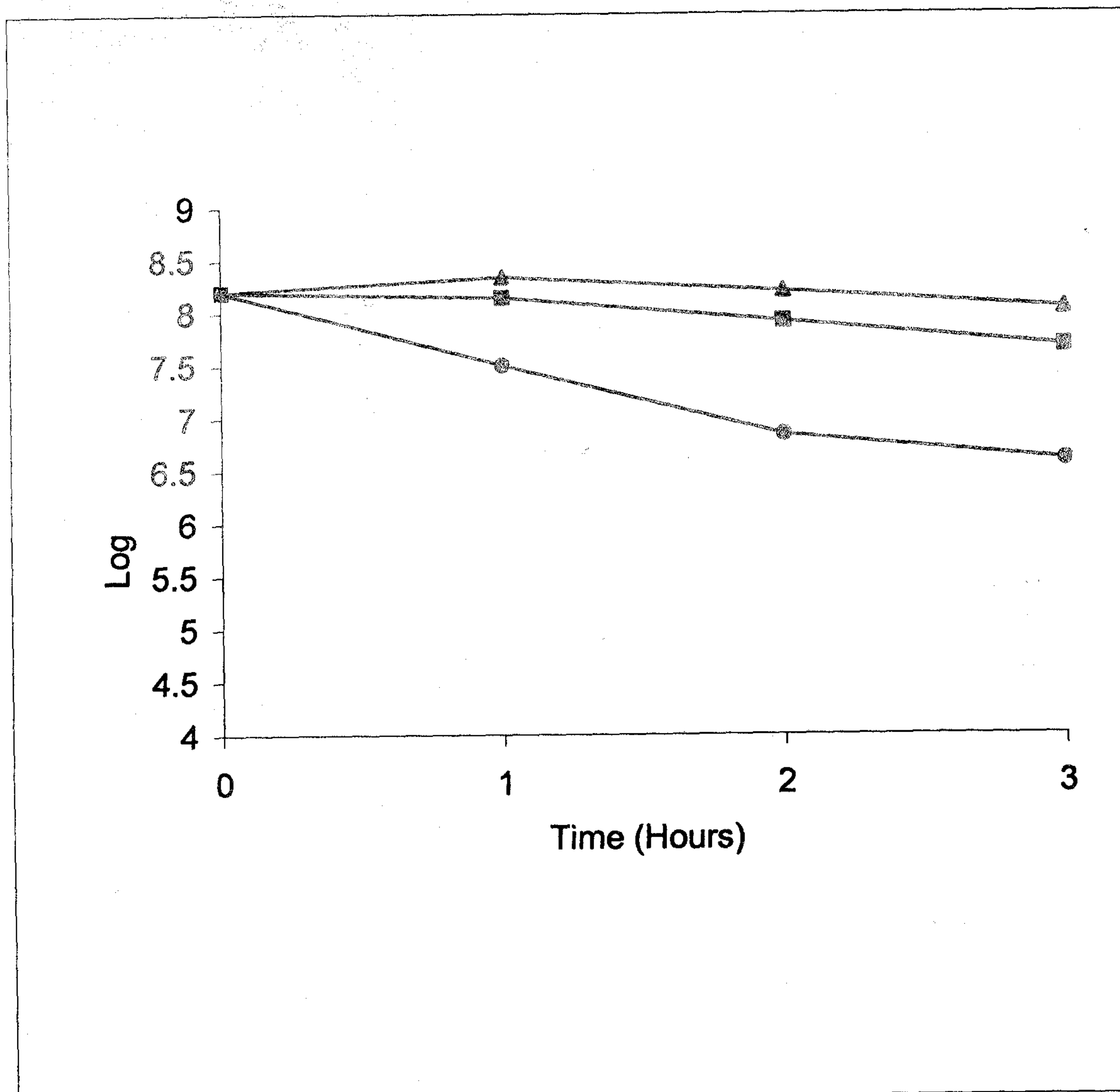

Fig. 4: Effect of antistaphylococcal metabolite from *A. pullulans* NI. 3 on viability of *S. aureus*
S. aureus cells were added in 10 ml of 25 mM phosphate buffer (▲), phosphate buffer containing 100 µl of ethanol (■) and phosphate buffer containing 5 U/ml of antimicrobial metabolite (• 5U/ml) and incubated for the indicated period at room temperature and total viable counts (TVC) were determined.

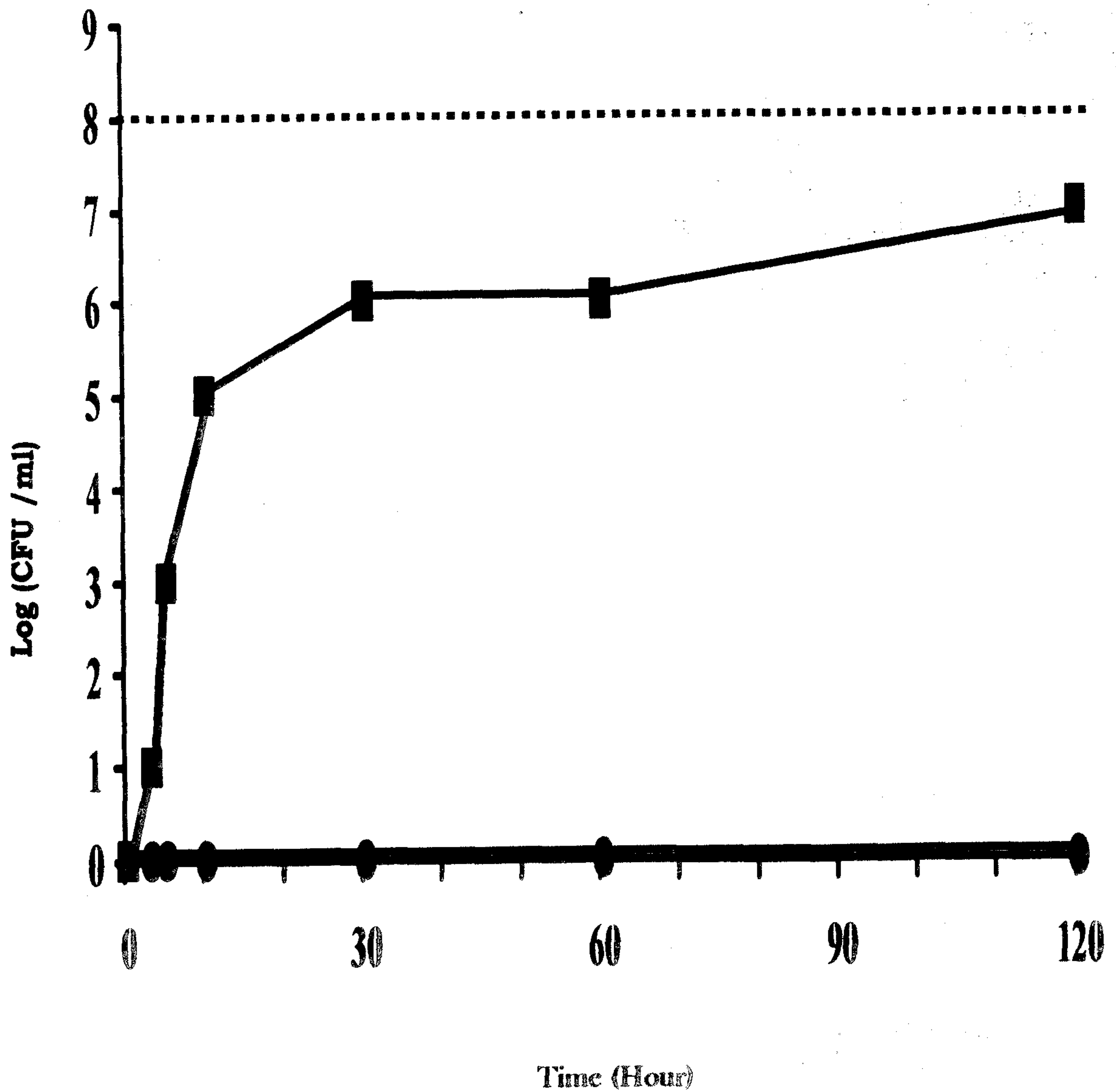

Fig. 5: Kinetics of potassium efflux from *S. aureus* in presence of antimicrobial metabolite
S. aureus **cells were suspended in 25 ml of 2.5 mM Na- HEPES buffer (▲), Na-HEPES buffer containing antimicrobial metabolite (6.24 units/ml, ●), incubated for two hours at room temperature. Five ml of sample was withdrawn at 30 min intervals and centrifuged at 12000 rpm for 10 min. The potassium content of supernatant was determined by flame photometer. Maximum potassium ion released from cells heated at 100°C for 10 minutes is shown by dotted lines (- - -).**

DISCUSSION

In the past several years, the field of chemotherapy had witnessed both the emergence and rapid spread of resistance in microorganisms. A significant percentage of clinical isolates, including species of streptococci, staphylococci and enterococci, are resistant to commonly used antibiotics, such as the new β- lactams and glycopeptides (9-11). Factors such as these make the search for newer antistaphyloccocal metabolite(s) imperative and essential. Moreover, significantly large numbers of microorganisms are yet unexplored for their capacity to give mankind the potential antistaphylococcal compounds of desired properties. *A. pullulans* NI.3, which is a safe and cosmopolitan organism (12), produced intracellular antistaphylococcal metabolite that inhibits growth of *Staphylococcus spp.* The effect of antistaphylococcal metabolite from *A. pullulans* on Gram-positive bacteria has not been investigated extensively. Our results on production of antistaphylococcal metabolite are encouraging for elimination of human infections.

The results presented in this paper clearly suggest that *Aureobasidium pullulans* NI.3 strain accumulated intracellular alcohol extractable metabolite, capable of inhibiting different *Staphylococcus spp.* The intracellular antistaphylococcal metabolite of *A. pullulans* NI.3 was moderately heat sensitive and active over a wide rage of pH. Proteolytic treatment of metabolite did not abolish its antistaphylococcal activity against *S. aureus.* It is therefore tempting to propose the presence of non-proteinic compound, which has potent antistaphylococcal activity. However, failure of proteolytic enzymes to inactivate antifungal metabolite produced by *A. pullulans* is not unusual (13). Also, treatment of antistaphylococcal metabolite from *A. pullulans* NI.3 to various detergents provided full recovery of antistaphylococcal activity.

Our data show that addition of antistaphylococcal metabolite to *S. aureus* cells results in an immediate loss of cellular K^+. These results demonstrate that the cytoplasmic membrane is the primary target for antistaphylococcal metabolite from *A. pullulans* NI.3. Therefore, the staphylocidal effect of the metabolite is most likely due to the formation of pores in the cytoplasmic membrane. A potential use of *A. pullulans* and its metabolite need more studies including further purification, mass spectra, nuclear magnetic resonance (NMR) and evaluation of toxicity are needed and in progress for confirmation of this suggestion.

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