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Optimization of Subtilin Production by *Bacillus Subitilis*

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ABSTRACT: In Pharmaceutical industries, several peptide antibiotics of importance are produced by *Bacillus subtilis*. Many antibiotics of importance are produced by *B.subtilis* species. Antibiotics are the biochemical secreted by microorganism which in low concentration inhibit the growth or kill other microorganism i.e. the antibiotics are "antimicrobial agent of microbial agent". The antibiotics producers are widely spread in nature, where they play very important role in regulating the microbial population of soil, water, sewage, and compost. During this study antibiotic production ability of microorganism was detected by conventional method of crowded plate technique. Further identification and isolation of the antibiotic was carried out by the technique of paper chromatography and Bioautography[1] and subtilin was identified to inhibit the growth of *S.aureus.* For the maximum production of the antibiotic the suitable parameters are optimized experimentally i.e. time, temperature, pH, media (carbon and nitrogen source).

KEYWORDS: Antimicrobial Compound; *Staphylococcus aureus; Bacillus subtilis;* Subtilin.

INTRODUCTION

Most bacteria produce antimicrobial compounds such as broad spectrum classical antibiotics, metabolic products viz. organic acids and lytic agents such as lysozyme. In addition, several types of protein exotoxin and bacteriocins, which are biologically active peptide moieties with bactericidal action, are also released[2]. Members of the Bacillus genus are generally found in soil and most of these bacteria have the ability to disintegrate protein (proteolytic activity). The high proportion of antimicrobial compound producing strains may be associated with ecological role, playing a defensive action to strains into an established microbial community[3]. When assayed against *Staphylococcus aureus* the culture supernatants produced zones of inhibition. Supernatant was further assayed for the identification of antibiotic by paper chromatography followed by Bioautography and the antibiotic from *Bacillus subtilis* found by bioautography in a position characteristic of subtilin.

S.aureus is a Gram-positive, spherical bacterium (coccus). *S.aureus* appears in clusters, like bunches of grapes. Growing in food, some strains can produce toxins which cause acute gastro-intestinal diseases if ingested. The organism can grow both with and without oxygen (facultatively anaerobic), and is catalase-positive and oxidasenegative. Virtually all *S.aureus* strains produce the enzyme coagulase. The main reservoirs of *S.aureus* are humans and animals. Healthy people carry the organism in their nose and

throat (50 %), on their hands (5-30 %), and in wounds. *S.aureus* can also colonise food contact surfaces, and it can become a persistent organism in slaughterhouses. *S.aureus* can contaminate foods through contact with contaminated hands, materials and surfaces, but also via the air (coughing).

The endospore-forming[4] rhizobacterium *Bacillus subtilis* – the model system for Gram-positive organisms, is able to produce more than two dozen antibiotics with an amazing variety of structures[5]. However, the disease-causing Bacillus species are now easily distinguishable from the helpful strains such as *Bacillus subtilis*. *B.subtilis* is beneficial is many ways, including industrial applications. It is used to produce a variety of enzymes, including amylase, which is helpful in the de-sizing of textiles and starch modification for the sizing of paper. *B.subtilis* also produces the enzyme protease, including subtilisin, which is used in detergents and the leather industry. Perhaps more notably, *B.subtilis* is used to produce many antibiotics, such as difficidin, oxydifficidin, bacilli, bacillomyin B, and Bacitracin, which is helpful in treating bacterial skin infections and preventing infection in minor cuts and burns. *B.subtilis* is also used as a fungicide. The bacteria colonize the root system, leaving no room for fungal disease organisms. It is used on agricultural seeds of vegetables, soybeans, cotton, and peanuts and on flower and ornamental seeds. It is also being used to produce insect toxins, including one to kill malarial mosquito larvae. According to a Toxic Substances Control Act report from the Environmental Protection Agency, *Bacillus subtilis* "is considered a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants.

Subtilin: The subtilin protein has the bactericidal effect on many Gram positive and certain Gram negative bacteria. This study is taken with the objective of isolation of *B.subtilis* from the soil and to assess the antibacterial effect of "subtilin". Subtilin production in *B.subtilis* is regulated in a growth-phase-dependent manner, starting in mid exponential growth phase and increasing to reach maximal level at the beginning of the stationary phase. Subtilin is a type I antimicrobial peptide (AMP) or lantibiotic that is produced by *B.subtilis*. Lantibiotics are peptide-derived antibiotics with high antimicrobial activity against various Gram-positive bacteria, including pathogenic bacteria such as propionibacteria, staphylococci, clostridia, enterococci and streptococci. This family of AMPs is characterized by the presence of unusual amino acids. Subtilin, a 32-amino-acid pentacyclic lantibiotic is structurally related to the widely utilized biopreservative nisin[6].

MATERIALS & METHODS

SCREENING OF ANTIBIOTIC PRODUCERS:

Antibiotic production ability of microorganisms can be detected by conventional crowded plate technique, in which soil or other source of microorganisms is diluted. The dilution selected in such a way that it should give 300-400 colonies and spread over nutrient medium. Colonies producing antibiotics are indicated by an area of agar around the colony that is free of growth of other colonies such colonies are selected for further use. Nutrient agar plates were prepared (composition for 1000ml (pH: 7.2 to 7.4) Beef extract 5 gm, Peptone 5 gm, Sodium Chloride 3 gm, Agar agar 15). The antibiotic producers *B.subtilis, B.megaterium, B.thurengienesis, Penicillin crysogenum* and test organisms *Pseudomonas, S.aureus, B.pumilis, Micrococcus luteus* were grown in nutrient broth for 4 days at 30^{o}C. Active culture of *S.aureus* was spreaded on the nutrient agar plates to get confluent growth and on the same plate active culture of *B.subtilis, B.megaterium, B.thurengienesis*, etc were spot inoculated. Similar procedure was done for the other test organism and plates were incubated at 30°C for 48 hours and observed zone of inhibition was measured.

IDENTIFICATION OF ANTIBIOTIC BY BIOAUTOGRAPHY:

One directional paper chromatography followed by Bioautography has been proved useful for differentiating antibiotics produced by *Bacillus* species in connection with a screening program. For purposes of comparison with unidentified antibiotics, *Rf* values have been determined with a number of solvents for most of the available Bacillus antibiotics active against gram positive bacteria and several of the polypeptidic antibiotics produced by other microorganisms. Multiplicity of antibiotic production obviously complicates attempts to identify antibiotics in un-fractionated material. However, even in whole culture similarities or difference between known and unidentified antibiotics may be noted by means of chromatography. Ascending chromatography on Wattman no .1 paper were developed in a glass jar containing 1 cm. of solvent mixture. Solvent compositions are based on volume measurement of each component. Effects of changes in solvent concentration were sufficiently large to warrant preparation of fresh mixture for each use. Samples were spotted 2 cm above the base of the paper, and placed in the solvent. After the solvent had migrated to the top of the paper the chromatograms were dried thoroughly. The migrated antibiotics were detected Bioautographically. Papers were placed for one half hour on nutrient agar plate's seed with the test organism *S.aureus*. The plates were incubated at 30°C for 24 hrs. As per the result obtained by the procedure of Bioautography the following solvent systems were changed as mentioned for further separation and identification of antibiotic

1. Solvent system 1: For 10 ml (7.4: 0.3: 2.5)
t-butyl alcohol: acetic acid: water
2. Solvent system 2: For 10 ml (6.5: 0.3: 3.2)
t-butyl alcohol: acetic acid: water
3. Solvent system 3: For 10 ml (2.5: 2.5: 0.3: 4.7)
n-butyl alcohol: ethyl alcohol: acetic acid: water
4. Solvent system 4: For 10 ml (5.5: 0.6: 4.0)
t-butyl alcohol: acetic acid: water
5. Solvent system 5: For 10 ml (7.0: 0.6: 2.4)
t-butyl alcohol: acetic acid: water
6. Solvent system 6: For 10 ml (4.0: 1.0: 5.0)
Alcoholic phase from n-butyl alcohol: acetic acid: water
7. Solvent system 7: For 10 ml (6.0: 1.3: 1.7)
Acetone: acetic acid: waters

OPTIMIZATION OF FERMENTATION CONDITION FOR ANTIBIOTIC PRODUCERS

To obtain higher level of antibiotic production from the actively growing culture of *B.subtilis* against gram positive *S.aureus* certain parameters such as time, temperature, pH, along with media were optimized.

I. Time course for antibiotic producers

For time optimization 100ml of starch broth was inoculated with 1ml of actively grown culture of *B.subtilis* and the broth was incubated at 30°C. From this after every 24 hours 2.0 ml of broth was collected aseptically, centrifuged at 10,000rpm for 15 minutes. Then pellet was discarded and supernatant was collected in a fresh tube and antibiotic activity in supernatant was checked by bioassay. For bioassay, the test organism *S.aureus* was spreaded on the surface of nutrient agar on this well of 2mm diameter were punched

using sterile stainless steel borer. To these wells, 15µl of supernatant were poured of each day in different well. Plates were incubated at 37°C for 24 hours and observed for the zone of inhibition. Diameter of zone of inhibition was recorded.

[Note: Diameter of zone of inhibition is directly proportional to the amount of antibiotic present in the supernatant.]

Sr. no	Antibiotic producer / Test organism	*Bacillus subtilis*	*Bacillus megatarium*	*Bacillus thuringenesis*	*Penicillin crysogenum*
1.	*S.aureus*	+	+	-	-
2.	*M. lutes*	-	+	-	-
3.	*P. aeruginosa*	+	+	-	-
4.	*Bacillus pumilis*	-	-	-	-
5.	*E. coli*	-	+	-	-

Table 1 Screening of Antibiotic Producers

(+): zone of inhibition (antibiotic production)

(-): no zone of inhibition (no antibiotic production)

Solvent no	Solvent system	Ratio (in 100 ml)	Rf value
Solvent 1	t-butyl alcohol: acetic acid: water	74 : 3 :25	0.41
Solvent 2	t-butyl alcohol: acetic acid: water	6.5 : 0.3 : 3.2	0.51
Solvent 3	n-butyl alcohol: ethyl alcohol : acetic acid: water	2.5:2.5:0.3:4.7	0.09
Solvent 4	t-butyl alcohol: acetic acid: water	5.5 : 0.6 : 4	0.33
Solvent 5	t-butyl alcohol: acetic acid: water	7.0 : 0.6 : 2.4	a) 0.22 b) 0.47
Solvent 6	Alcoholic phase from n-butyl alcohol: acetic acid: water	4.0 :1.0: 5.0	0.67
Solvent 7	acetone: acetic acid: water	6.0 : 1.3 : 1.7	0.0

Table 2 Identification of Antibiotic By Paper Chromatography and Bioautography

II. Effect of temperature on antibiotic production:

For optimization of temperature six sets of 20ml of starch broth were inoculated with 2% of actively growing culture of *B.subtilis*. All flasks were incubated at different temperatures viz, 28°C, 31°C, 34°C, 37°C, 41°C, room temperature for 96 hours (optimum incubation time). After incubation broth was centrifuged at 10,000rpm for 15 minutes. Then pellet was discarded and supernatant was collected in fresh tube and antibiotic activity in supernatant was checked by bioassay.

III. Effect of pH on antibiotic production:

For pH optimization each flask of 20ml of starch broth with different pH viz, 4, 5, 6,7,8,9 was inoculated with 2% of actively growing culture of *B.subtilis*. All flasks were incubated at 28°C (Optimized temperature) and 96 hour (Optimized time). After incubation broth was centrifuged at 10,000 rpm for 15 minutes. Supernatant was collected in fresh tube and antibiotic activity in supernatant was checked by bioassay.

MEDIA OPTIMIZATION

Effect of carbon and nitrogen source:

Nitrogen source of 0.5% i.e. peptone was dissolved in distilled water and distributed in different flask. To each flask different carbon source of 1.0% i.e. wheat flour, rice flour, corn flour, glucose and starch was added. Similarly to see the effect of nitrogen Optimized carbon source i.e. corn flour was dissolved in distilled water and distributed in different flasks. To each flask different nitrogen source was added i.e. yeast extract, beef extract, peptone, tryptone, and urea was added and the pH was adjusted at 8 (Optimized pH) and media autoclaved. 2% of *B.subtilis* was inoculated to each flask of production broth and was incubated at 28°C (Optimized temperature) for 96 hour (Optimized time). After incubation, broth was centrifuged at 10,000rpm for 15 minutes at room temperature. Supernatant was collected in fresh tube and antibiotic activity in supernatant was checked by bioassay.

RESULTS & DISCUSSION

Sr. no	Time of incubation (in hrs)	Diameter of zone of inhibition (in cm)
1.	24 Hrs	0.00
2.	48 Hrs	0.80
3.	72 Hrs	1.00
4.	96 Hrs	1.64
5.	120 Hrs	1.30
6.	144 Hrs	1.00
7.	168 Hrs	0.80
8.	192 Hrs	0.00

Table 3 Time Course for Antibiotic Production

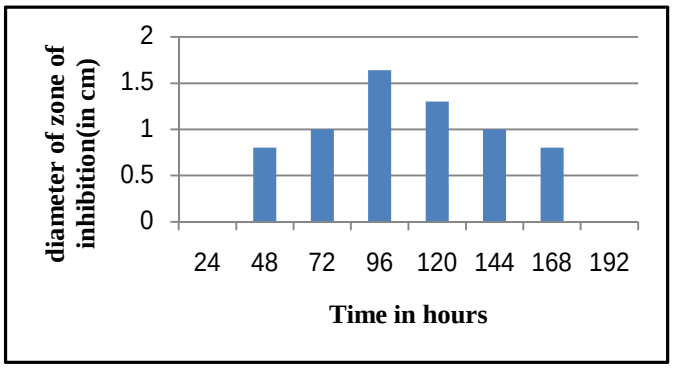

Figure 1 Time Course for Antibiotic Production

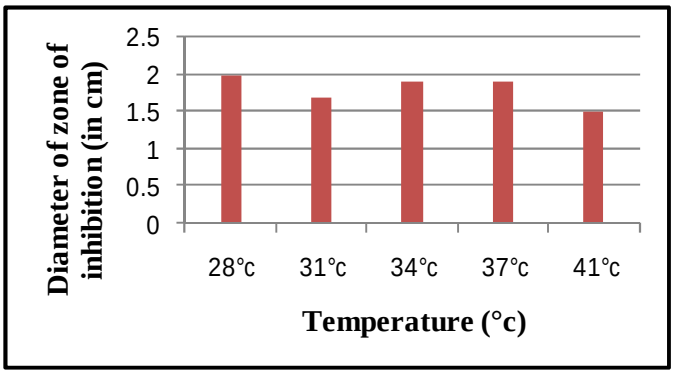

Figure 2 Effect of Temperature on Antibiotic Production

Sr. no	Incubation temperature (in °C)	Diameter of zone of inhibition (in cm)
1.	28°C	2.0
2.	31°C	1.7
3.	34°C	1.9
4.	37°C	1.9
5.	41°C	1.5

Table 4 Effect of Temperature on Antibiotic Production

Sr. no	Carbon source	Diameter of zone of inhibition (in cm)	Nitrogen source	Diameter of zone of inhibition (in cm)
1.	Wheat flour	1.8	Beef extract	1.80
2.	Rice flour	1.6	Yeast extract	2.00
3.	Corn flour	2.0	Peptone	3.96
4.	Starch	1.4	Tryptone	2.03
5.	Glucose	0.0	Urea	1.93

Table 5 Media optimization effect of carbon and nitrogen source

Sr. no	pH	Diameter of zone of inhibition (in cm)
1.	4	1.3
2.	5	1.7
3.	6	1.6
4.	7	1.5
5.	8	1.9
6.	9	1.5

Table 6 Effect of pH on antibiotic production

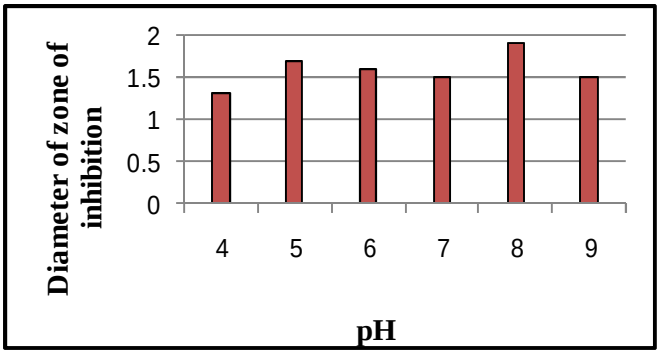

Figure 3 Effect of pH on antibiotic production

Bacillus subtilis and *Bacillus megatarium* were found to produce antibiotic compound which were inhibiting growth of *S.aureus, P. aeruginosa*. Of this *B.subtilis* was used for further studies. The antibiotic was not able to inhibit the growth of *M. luteus, B.pumilis,* and *E. coli*. By observing the Rf values obtained by subsequent paper chromatography using different solvent system having organic solvents at different concentration followed by Bioautography it was observed that the antibiotic obtained is Subtilin. Effect of various parameters on the production of antibiotics was studied, which showed that the antibiotic production by *B.subtilis* started after 24 hrs of incubation and reached to maximum level after 96 hrs of incubation, after which the antibiotic concentration reduced. After 192 hrs, no antibiotic was detected in fermentation broth. Hence optimum time for antibiotic production was 96 hrs. The optimum temperature for antibiotic production was found to be 28°C. The effect of medium composition was also studied in which it was observed that antibiotic production was found to be maximum when fermentation was carried out at pH 8 and production was comparatively higher when peptone was used as nitrogen source corn flour as carbon source.

CONCLUSION

The antibiotic production was carried out using *Bacillus* species i.e. *B.subtilis* and by screening procedure it was found that it could inhibit the growth of *S.aureus*. The diameter of zone of inhibition obtained in bioassay was 5.3cm. Further identification and isolation of antibiotic was carried out using the techniques of paper chromatography and Bioautography and Subtilin was identified to inhibit the growth of Gram positive *S.aureus*. For the maximum production of antibiotic the suitable parameters were optimized experimentally i.e. time (96 hrs i.e. 4 days), temperature (28ºC) and pH (8) along with media optimization i.e. carbon and nitrogen source.

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