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Research Article

Study on Production of Extracellular Amylase from *Bacillus subtilis* Strain KPA under Mild Stress Condition of Certain Antimicrobials

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ABSTRACT
The present study was investigated to improve *Bacillus subtilis* strain KPA for extracellular amylase production under mild stress condition of certain antimicrobials. The novel isolated bacterial culture was exposed to different concentrations of *Allium sativum*, ampicillin and Mercuric chloride at their sub- MIC values for 24 to 72 h. Exposure of *Allium sativum* and ampicillin to strain KPA did not give improved extracellular amylase production. *Allium sativum* and ampicillin at the concentration of 250 µ l and 100 µ l were able to induce strain KPA for amylase production of 1896.257 U/ml/min and 1740.976 U/ml/min respectively after 48 h of incubation. On the other hand Mercuric chloride at the concentration of 250 µ l was able to improve strain KPA even after 24 h of incubation for maximum enzyme production (2024.136 U/ml/min) compared to the enzyme produced (1854.099 U/ml/min) by the parental strain. The extracellular enzyme produced by strain KPA under mild stress condition of certain antimicrobials tested here were 2-3 fold higher than that of previously isolated Bacillus strains. The results clearly conclude the role of novel isolated strain in various industries such as textile, paper, pulp, food etc. due to enhanced production of amylase under mild stress conditions.

Key words: *Allium sativum*, Ampicillin, Amylase, *Bacillus subtilis*, Mercuric chloride, sub- MIC.

INTRODUCTION

Amylases are among the most important enzymes used in biotechnology, particularly in process involving starch hydrolysis, industries such as brewing, food, paper, textile and pharmaceuticals. Alpha amylase is an extra cellular enzyme, which is used in the starch processing industry where it breaks starch into simple sugar constituents[1]. Amylases are among the most important enzymes and account for about 30% of the world's enzyme production[2]. They can be obtained from several sources, such as plants, animals and microorganisms. Microorganisms are important producers of industrial enzymes, due to their diversity. Production of industrially important enzymes from eukaryotes has been replaced with microorganisms. Bacterial amylases could be potentially useful in the pharmaceutical and chemical industries. Although many microbial sources are available for producing amylases, the capacity of *Bacillus strains* to produce large quantities of enzymes has placed them among the most important industrial enzyme producers. The genus Bacillus produces a large range of industrial important extracellular enzymes (amylases and proteases)[3]. *B. amyloliquefaciens*[4] and *B. subtilis*[5] are industrially important *Bacillus* species. Uses of amylase in various industries have placed greater stress on increasing indigenous amylase production and search for more efficient processes. Bacteria launch stress responses (chaperones) under hostile and challenging environmental conditions like during depletion of nutrients, oxygen, changes in temperature and presence of antimicrobial agents. In harsh condition, bacteria have developed special physiologic mechanisms including the production and secretion of specific proteins and enzymes. Hence the production of enzymes was higher than the normal conditions. Bacteria under stress "switch on" a catalogue of genes responsible for enhanced production of enzymes. Antimicrobials are one of the stresses in the bacterial world. The antimicrobial agents may be natural plants products, antibiotics, heavy metals etc. The production of microbial alpha amylase by bacteria is dependent on the type of strain, composition of medium, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, time of incubation and thermostability[6]. Considering the industrial importance of amylase, the purpose of the current investigation was to screen novel strain of *Bacillus* species isolated from poultry farm and to enhance extracellular amylase production by the novel bacterium in presence of certain antimicrobials such as *Allium sativum*, ampicillin and Mercuric chloride.

MATERIALS AND METHODS

Sample collection, Isolation and Screening: Poultry faeces sample was collected from poultry farm of Guduvanchery, Tamil Nadu (India). Faeces sample was brought to the

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Table 1: Shows Morphological and Biochemical test reports of strain KPA

S.no.	Tests	Result
1	Morphology	Rod shaped
2	Gram Staining	Positive
3	Motility	Positive
4	Indole	Negative
5	Methyl Red	Negative
6	Voges –Proskauer	Positive
7	Citrate utilization	Positive
8	Urease	Negative
9	Catalase	Positive
10	Amylase	Positive
11	Oxidase	Negative
12	Endospore staining	Endospores observed

Table 2: Shows MIC and sub- MIC values for antimicrobials against strain KPA

Antimicrobials	MIC value (%)	Sub- MIC value (%)
Allium sativum	10	5
Ampicillin	10	5
Mercuric chloride	20	10

laboratory in aseptic condition. A serial dilution of the sample (1 gram of faeces soil) was made using sterile saline until a dilution of 10-6 was obtained. 100 µ l of this dilution was spread over nutrient agar petriplates and incubated at 37ºC for 24 hours. Pure culture was isolated and subcultured in the same medium at 37ºC. The culture was streaked and kept in incubator at 37°C for 24 hours and was preserved in slants at 4±2°C.

Morphological and Biochemical tests: Purified isolate was characterized by Biochemical analysis using Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test, Urease test, Oxidase test and Amylase test (according to the Bergey's Manual of Systemic Bacteriology). Gram staining, Endospore staining and Motility test were performed under Morphological tests.

Isolation of Genomic DNA: Two ml of bacterial culture were centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded. One ml of UniFlexTM Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 minutes at 37ºC in a water bath. To the lysed samples 1 ml of 1:1 phenol:chloroform were added and mixed well. The samples were centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlexTM Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant was discarded. The pellet was air dried for about 10-15 minutes till the ethanol evaporates. The pellet was resuspended in 50-100 µl of UniFlexTM Elution Buffer. DNA was stored at -20ºC.

Amplification of 16S rRNA genes by PCR, Sequencing and Alignment: The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with Taq DNA polymerase and primers 27F (5` AGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACGGCTACC TTGTTACGACTT 3`). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/.

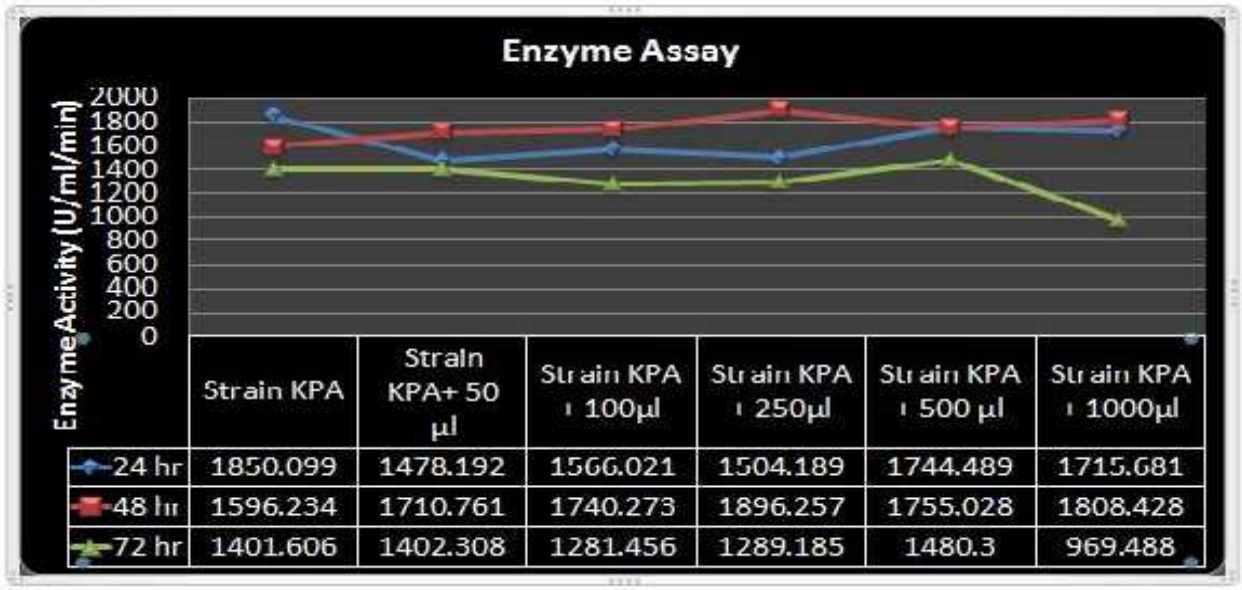
Antimicrobials of interest: *Allium sativum* (Spice), ampicillin (Antibiotic) and Mercuric chloride (Heavy metal) were used to provide stress to the novel bacterial strain.

Sample preparation: *Allium sativum* (Garlic) was purchased from local market of Nungambakkam, Tamil Nadu (India). The garlic bulbs without the outer skins were grinded in a sterilized mortar and pestle. The fine garlic mesh was centrifuged at 6000 rpm for 10 minutes. The supernatant was filter sterilized by using a 0.2 µ m syringe filter to produce sterile supernatant. Heavy metal salt solution was prepared by mixing Mercuric chloride (HgCl2) in sterilized distilled water at the concentration of 25 mg/L. Ampicillin (10 µ g) was prepared by mixing appropriate volume of DMSO.

MIC and sub- MIC determination of antimicrobials: MIC and sub- MIC values of *Allium sativum* juice, Mercuric chloride and ampicillin were determined by Microdilution method 7. Serial dilutions of *Allium sativum* juice, Mercuric chloride and ampicillin were prepared from 100% to 1% concentration. Serial dilutions of antimicrobials were added to bacterial culture in Microtitre plate. The plate was incubated at 37ºC for overnight. The bacterial growth was detected by the addition of 3-(4, 5-dimetylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solutions (10 mg/ml) in the wells. The highest dilution of antimicrobials inhibiting the bacterial growth was considered as MIC value. Half of the value of MIC was considered as sub- MIC value.

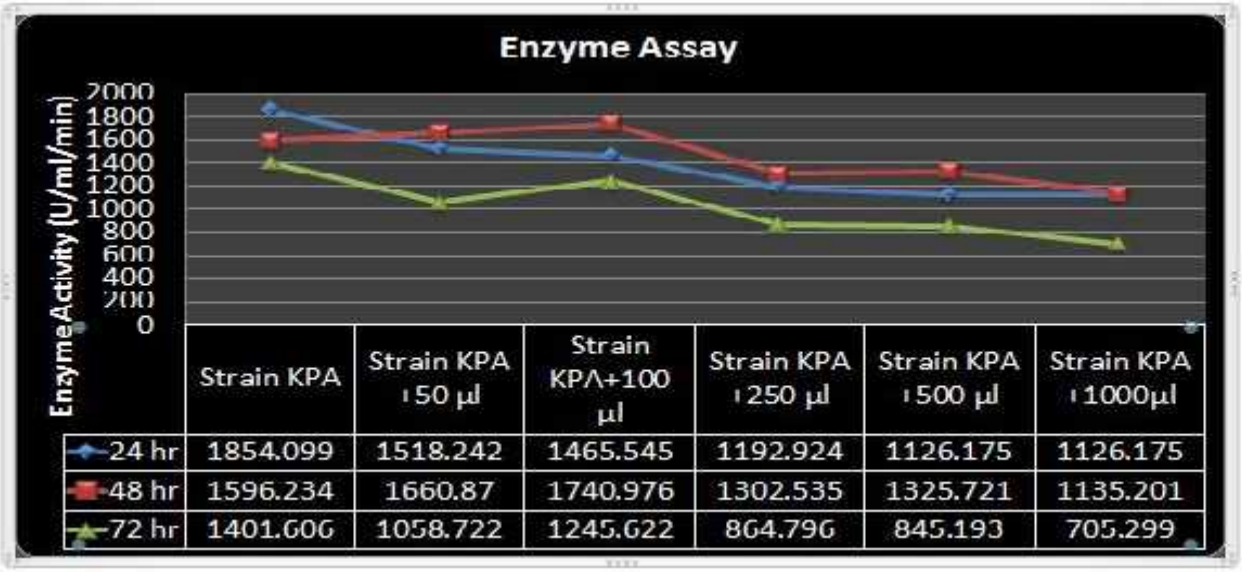
Inoculum preparation: Fifty millilitre of inoculum medium containing nutrient broth (pH- 7.0) was transferred to 250 ml of conical flask and cotton plugged. The flask was sterilized at 121ºC, 15 lb pressure for 15 minutes. A loopfull of bacteria was inoculated aseptically into the bacteria contained in genomic database banks, using the NCBI BLAST. The comparison showed that the similarity cooled medium and kept for incubation overnight at 37ºC in a rotatory shaker.

Shake flask fermentation: Extracellular amylase fermentation was carried out in 16 conical flasks (volume capacity 250 ml), each flask containing 50 ml of Nutrient



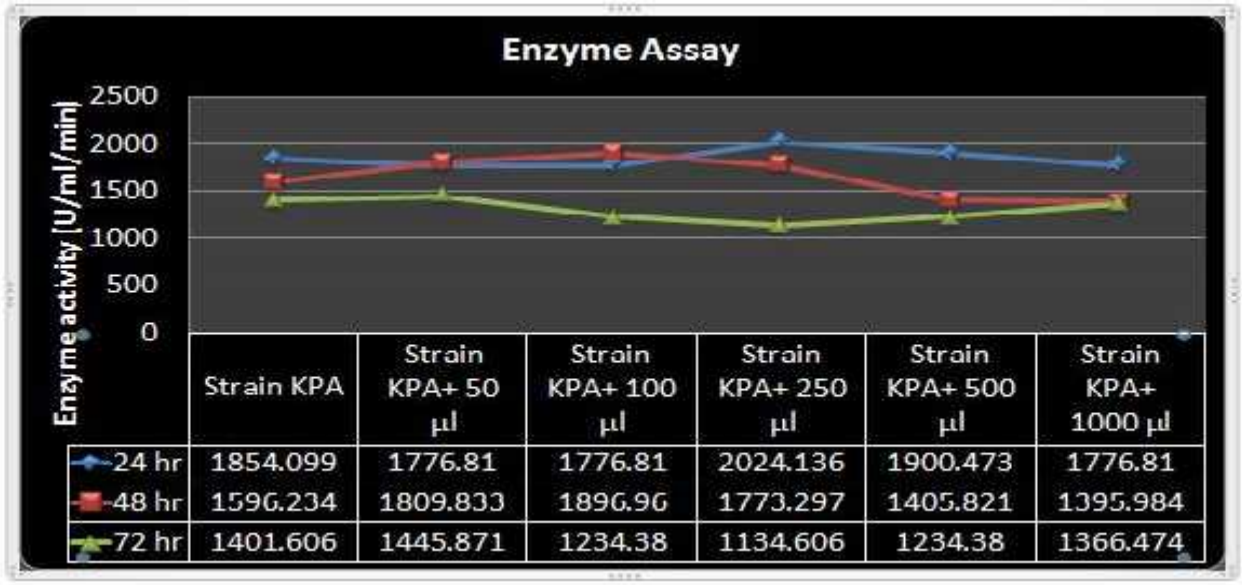
	Strain KPA	Strain KPA+ 50 µl	Strain KPA + 100µl	Strain KPA + 250µl	Strain KPA + 500 µl	Strain KPA + 1000µl
24 hr	1850.099	1478.192	1566.021	1504.189	1744.489	1715.681
48 hr	1596.234	1710.761	1740.273	1896.257	1755.028	1808.428
72 hr	1401.606	1402.308	1281.456	1289.185	1480.3	969.488

Fig. 1: Shows Amylase activity of strain KPA in presence of Allium sativum



	Strain KPA	Strain KPA + 50 µl	Strain KPA+100 µl	Strain KPA + 250 µl	Strain KPA + 500 µl	Strain KPA + 1000µl
24 hr	1854.099	1518.242	1465.545	1192.924	1126.175	1126.175
48 hr	1596.234	1660.87	1740.976	1302.535	1325.721	1135.201
72 hr	1401.606	1058.722	1245.622	864.796	845.193	705.299

Fig. 2: Shows Amylase activity of strain KPA in presence of Ampicillin



	Strain KPA	Strain KPA+ 50 µl	Strain KPA+ 100 µl	Strain KPA+ 250 µl	Strain KPA+ 500 µl	Strain KPA+ 1000 µl
24 hr	1854.099	1776.81	1776.81	2024.136	1900.473	1776.81
48 hr	1596.234	1809.833	1896.96	1773.297	1405.821	1395.984
72 hr	1401.606	1445.871	1234.38	1134.606	1234.38	1366.474

Fig. 3: Shows Amylase activity of strain KPA in presence of Mercuric chloride

Broth and 1% Starch. The flasks were divided into 3 sets (each set containing 5 flasks). One flask was kept as control. One set was labelled as 50 µ l, 100 µ l, 250 µ l, 500 µ l and 1000 µ l of Allium sativum. Second set was labelled as 50 µ l, 100 µ l, 250 µ l, 500 µ l and 1000 µ l of ampicillin. Third set was labelled as 50 µ l, 100 µ l, 250 µ l, 500 µ l and 1000 µ l of Mercuric chloride. The flasks were sterilized at 121ºC for 15 minutes. Each flask was inoculated with 500 µ l of overnight bacterial inoculum. The flasks were kept in the rotatory shaker at 37ºC for 2-3 h (lag phase of novel strain). The flasks were taken out and each flask except control was inoculated with appropriate concentration of antimicrobials (from sub-MIC value) as labelled on the flasks. All the flasks were again kept in rotatory shaker for 24-72 h of incubation.

Cell-free supernatant preparation: The overnight bacterial cultures grown in presence of different concentrations of antimicrobials were centrifuged at 7000 rpm for 10 minutes. The control flask containing only the bacterial culture was also centrifuged at the same rpm. The supernatants were collected and extracellular amylase assay was performed. The same procedure was repeated till 72nd h.

Enzyme Assay: Amylase was estimated according to the method of Rick and Stegbauer (1974) 8. One millilitre of enzyme extract was added to a test tube containing 1.0 ml of 1.0 % starch solution. The mixture was incubated at 60ºC for 10 min. After the incubation, 1.0 ml of DNS reagent was added to each of the tubes. The tubes were placed in boiling water for 5 min and cooled to room temperature. The contents of tubes were diluted up to 10 ml with sterilized distilled water. The optical density (OD) of reaction mixture was determined at 540 nm. One unit of activity is equivalent to that amount of enzyme, which in 10 min liberates reducing group from 1.0 % Lintner' s soluble starch corresponding to 1.0 mg maltose hydrate.

RESULTS

Isolation and identification of new strain of bacteria: The morphological and biochemical characteristic of the isolate were studied (Table 1). The isolated bacterial strain was identified as *Bacillus* sp. based on the taxonomical characteristics. Genomic DNA of the isolate was visualized under UV. The amplicon of 483 bp was observed using PCR amplification. In the present study, 16S rRNA gene sequencing of the isolate was investigated. The isolate was identified as *Bacillus subtilis* strain KPA by comparing the similarity with the reference species of of 16S rRNA gene sequences was 99%. The identities of strain KPA were determined by comparing them with the available sequences of the strains and with high scored rRNA sequences in BLAST search. The novel isolated sequence was deposited in Genbank (Accession number-KC918878), maintained by NCBI, USA.

MIC and sub-MIC determination of *Allium sativum*, ampicillin and Mercuric chloride: The effects of antimicrobial on bacterial cell growth were studied to determine the minimal inhibition concentration (MIC) value which could inhibit the bacterial growth. In this experiment, the bacterial viability was evaluated by using MTT solution as an indicator. The results from Table 2 indicated that the MIC values of *A. sativum*, ampicillin and Mercuric chloride on *B. subtilis* strain KPA was 10%, 10% and 20% respectively. The sub-MIC values (which is 0.5 x MIC) for each treatments was further calculated as 5%, 5% and 10% for *A. sativum*, ampicillin and Mercuric chloride respectively.

Quantitative analysis of extracellular amylase production: The enzyme production by strain KPA was increased from 1850.099 U/ml/min to 1896.257 U/ml/min after 48 h in presence of 250 µ l of *Allium sativum.* There was decrease in the production of amylase by stressed bacteria compared to normal during 24 h of incubation. The enzyme production was again decreased after 72 h. Amylase production by control bacteria was successively decreased after regular interval of time but the bacteria under the stressed condition of *Allium sativum* were able to produce more enzymes after 48 h compared to the 24 h. *Allium sativum* stress to the bacteria did not end up in a high yielding production of Amylase (Fig.1).

Bacillus subtilis strain KPA was not able to produce more extracellular amylase in presence of ampicillin. The bacteria under stress condition of ampicillin were producing 1740.976 U/ml/min (after 48 h) of amylase. On the other hand the parental strain was able to produce 1854.099 U/ml/min of enzyme even after 24 h of incubation. But the enzyme production by stressed bacteria was more than that of control bacteria after 48 h (Fig. 2). The bacteria were able to produce more amount of amylase in presence of Mercuric chloride stress. The enzyme production by the bacteria was more compared to the production under the stress of *Allium sativum* and ampicillin. The bacteria under Mercuric chloride stress (250 µ l) were able to produce 2024.136 U/ml/min of amylase compared to the parental strain (1854.099 U/ml/min) even after 24 h of incubation. After 48 h of incubation the enzyme production was decreased but it was more than that of control or parental bacteria. The enzyme production was again decreased after 72 h of incubation. The bacteria under mild stress condition of Mercuric chloride were able to enhance the production of extracellular enzyme (Fig. 3).

DISCUSSION

In the present study, *Bacillus subtilis* strain KPA was improved for amylase production. *Allium sativum* and ampicillin stress did not end up in a high yield production of extracellular enzyme. But stress in presence of Mercuric chloride was able to enhance the production of extracellular enzyme compared to the parental strain. Previous reports have employed random mutagenesis for alpha amylase production by exposing the cultures with UV or chemicals like EMS and nitrous acid[9]. *Bacillus subtilis* strain KPA was able to enhance the production of extracellular amylase in presence of antimicrobials after 48 h of incubation. Microbial secondary metabolites, including microbial enzymes are usually not produced during the phase of rapid growth (log phase), but are synthesized during a subsequent production stage (stationary phase), which is when primary nutrient source

is depleted[10]. A culture of *B. subtilis* cells that has exhausted one or more essential nutrients and under stress will enter the stationary phase of growth. At this stage, the production of extracellular proteins and enzymes as well as antibiotics is induced. These various phenomena reflect the complex response of the cells to stress and show that a rapid adjustment to environmental change is essential for survival. The results of present study indicate that production of amylase was increased in strain KPA in presence of antimicrobials after 48 h of incubation. During 24 h of incubation bacteria were not able to adapt according to the stress conditions provided resulting in less amount of enzyme production. But the bacteria in presence of mild stress condition of Mercuric chloride at particular concentration were able to produce more amount of amylase even after 24 h of incubation. This may be due to the different mode of action of Mercuric chloride at sub-MIC value on strain KPA. The stress conditions provided by Mercuric chloride to strain KPA may be different from other antimicrobials tested here, resulting in enhanced production of enzyme even after 24 h of incubation. Once the bacteria entered into the stationary phase the bacteria produced enhanced amount of amylase in order to overcome the stress response. Hence, it demonstrated that *B. subtilis* strain KPA cells were induced to produce amylase during stationary phase as an adaptation strategy in stress conditions caused by *A. sativum*, ampicillin and Mercuric chloride. In the present investigation *B. subtilis* strain KPA tend to secrete more enzymes after treating with *A. sativum*, ampicillin and Mercuric chloride. This may be due to the fact that *B. subtilis* lacks an outer membrane. Therefore, lots of extracellular enzymes are secreted directly into the growth medium. Extracellular and surface-associated proteins and enzymes play important role in adaptations of *B. subtilis* to the stress environment. *Bacillus* species secrete numerous enzymes which enabling them to degrade a variety of substrates and survive in a complex and continuously changing environment. *B. subtilis* secretes high levels of enzymes under poor nutrient conditions. It is supported by the previous study stated that the antimicrobials or antibiotics at sub-MIC can act as signaling molecule or inducer in the bacteria metabolite process by modulating their transcriptional machinery process[11]. Environmental changes occur often suddenly and therefore, a quick response is crucial to assure cell survival. In such context, translational regulation of pre-existing mRNAs provides a fast and efficient way to control gene expression. The production of extracellular amylase by strain KPA in presence of stress conditions was 2-3 folds compared to the previous findings. This may be due to the reason that the production of microbial amylase by bacteria is dependent on the type of strain, composition of medium, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, time of incubation, thermostability and the control of contamination during fermentation. The present study clearly demonstrated that economically valuable enzymes such as amylase can be commercially produced on large scale from novel isolated strain of *Bacillus* species under mild stress conditions.

CONCLUSION

This exploratory study showed that *B. subtilis* strain KPA could produce enhanced extracellular amylase (2- 3 folds) in presence of *A. sativum*, ampicillin and Mercuric chloride compared to the previously isolated *Bacillus* strains under stress conditions. The present findings led to the generalized assumption that antimicrobials at sub-MIC level were able to induce extracellular enzyme production by regulating the transcription machinery in certain bacterial strain. Economically valuable enzymes such as amylase can be commercially produced on large scale from novel isolated strain of *Bacillus* species. The results of present study clearly concluded that the novel isolated strain can play a major role as its biotechnological applications in various industries of textile, paper, food, bread making, detergents etc. Further study is necessary to know the rate of amylase production by this strain under mild stress conditions during log phase of bacterial growth. Another research should also be continued to determine the enzyme activity by optimizing the media and various parameters.

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