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# Production of Antimicrobial Agents by *Bacillus subtilis* Through Fermentation of Molasses

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**Abstract:** Antimicrobial agents were produced using specific strain of *Bacillus subtilis* by fermentation of molasses in medium containing $MgSO_4.7H_2O$, $CuSO_4.7H_2O$ and $KH_2PO_4$ under semi-shake conditions at 37EC (pH 7.2). Alluding to the individual effect of the salts against *E. coil* the same conc. of $MgSO_4.7H_2O$, (0.05%) had similar impact both after 48 hrs and 72 hrs incubation, while for the other two salts $lCuSO_4.7H_2O$ and $KH_2PO_4$), an increase in conc. concomitantly enhanced the antimicrobial activity only during delayed incubation. Similarly, against *Streptococcus pyogenes*, all the three salts had better results at delayed span of time (72 hrs). For dual salt combinations $MgSO_4.7H_2O/CuSO_4.7H_2O$ and $MgSO_4.7H_2O/KH_2PO_4$ in relative conc. of 0.005/0.1 and 0.005/0.5 against *S. pyogenes* and $MgSO_4.7H_2O/CuSO_4$ in conc. of 0.05/0.1 against *E. coil* yielded better effect after 72 hrs. incubation. In case of triple salt combinations too, 72 hrs incubation proved suitable for better yield of antimicrobial agents. Maximum antimicrobial activity was found at relative salt conc. of 0.005, 0.05 and 0.5% of $MgSO_4.7H_2O$, $CuSO_4.7H_2O$ and $KH_2PO_4$ respectively. The extract did not show remarkable antifungal activity against *Fusarium oxysporum.* No haemolytic activity of the broth extract was found against human erythrocytes.

**Key words:** Antimicrobial activity, bacillus subtilis, fermentation, molasses

## Introduction

Antibiotics are the substances produced by microorganisms that inhibit growth or kill bacteria and fungi (Waksmann, 1967). They possess inhibitory, static, degenerative, lysing or killing activities against animal and plant pathogens such as bacteria, viruses, rickettsia, actinomycetes, fungi, algae or protozoa.

These are used mainly in the fields of human and veterinary medicine, animal feeds, agriculture and food industry (Betina, 1983). The antibiotics are being attempted to be synthesized in large amounts throughout the world by using agro-industrial wastes. A developing country like Pakistan does not afford the huge import of antibiotics from abroad, costing a lot of money worth millions of rupees each year. Hence production of antibiotics can be tried through bacterial as well as fungal fermentations using cheaper agro industrial wastes like maize bran, wheat bran, rice husk, rice bran, gram bran, molasses etc. as substrates.

Molasses has been used to produce useful chemicals (Rale, 1990). In Pakistan, sugarcane cultivation occupies 0.96 million hectares of the total area under cultivation with production of 45.2 million tones (Anonymous, 1995-96). Total production of molasses becomes around 38.05 million tones per year (Anonymous, 1993). Therefore, this waste material can be exploited for the production of useful Materials.

*Bacillus subtilis* is an aerobic, gram positive, spore forming bacterium which can be easily grown on a variety of agricultural wastes for the production of antibiotics and various enzymes through fermentation.

In the present project the prime objective was to produce antimicrobial agents by *B. subtilis* through fermentation of molasses. The optimal conditions for maximum bacterial growth (so as to have maximal yield) were also standardized.

## Materials and Methods

The substrate (molasses) was obtained from Crescent Sugar Mills, Faisalabad. It was cleaned and kept in air tight containers. The complete proximate analysis of the substrate was made, following AOAC methods (Anonymous, 1984).

The specific strain of *B. subtilis* producing antibiotic was used to prepare test inoculum and the spore suspension was standardized for use by adjusting the spore concentration at $10^6$-$10^8$ spores/ml (Zarofonetis, 1959). Prior to the fermentation for production of antimicrobial agents, the physical and chemical conditions of the growth medium were optimized for optimal yields. The replicates with different salt combinations alongwith controls were run for fermentation as batch culture. Growth medium flasks after sterilization were inoculated with one ml standard spore suspension and later incubated at 37EC for 72 hrs. The fermented broth extracts from each of the experimental and control flasks were harvested by centrifugation at 3000 rpm for 15 minutes (Khan, 1983). The antimicrobial activity of fermented broth extracts so harvested was examined against a selective range of microorganisms comprising both fungi as well as bacteria such as *E. coli*, *streptococcus pyogenes*, *Fusarium oxysporum*.

Antibacterial activity was detected by disc diffusion method (Cruickshank *et al*., 1975) whereas antifungal property was assessed through spore germination test (Ahmed *et al*., 1992). The data were statistically evaluated using analysis of variance (Steel and Torrie, 1986).

## Results and Discussion

Molasses is a good source of antibiotics through fermentation technology by using *Bacillus subtilis* (Sommer, 1992). The proximate analysis of the molasses revealed that it contained following contents.

| | |
|---|---|
| Moisture | 25.63% |
| Ash | 20.29% |
| Ether extract | 0.335% |
| Crude Protein | 2.5% |

The extracts collected from fermented molasses replicates were subjected to antibacterial and antifungal activity.

Table 1: Effect of varying conc. of $MgSO_4.7H_2O$, $CuSO_4$, $7H_2O$, $KH_2PO_4$, on the antibacterial activity against *E. coli*

| Conc. of $MgSO_4.7H_2O$ (%) | Conc. of | Conc. of | 48 hours incubation | 72 hours incubation |
|---|---|---|---|---|
| 0.005 | 0.01 | 0.1 | 266 ± 0.41 | 0.02 ± 0.23 |
| | | 0.3 | 2.30 ± 0.32 | 2.33 ± 0.17 |
| | | 0.5 | 1.66 ± 0.31 | 1.33 ± 0.30 |
| | 0.05 | 0.1 | 1.66 ± 0.13 | 1.35 ± 0.11 |
| | | 0.3 | 1.65 ± 0.41 | 1.69 ± 0.18 |
| | | 0.5 | 1.02 ± 0.12 | 1.31 ± 0.04 |
| | 0.01 | 0.1 | 1.33 ± 0.41 | 3.00 ± 0.17 |
| | | 0.3 | 1.05 ± 0.62 | 3.00 ± 0.13 |
| | | 0.5 | 1.67 ± 0.47 | 2.33 ± 0.21 |
| 0.05 | 0.01 | 0.1 | 1.68 ± 0.14 | 2.31 ± 0.29 |
| | | 0.3 | 2.62 ± 0.22 | 3.00 ± 0.23 |
| | | 0.5 | 2.13 ± 0.53 | 2.00 ± 0.06 |
| | 0.05 | 0.1 | 3.00 ± 0.12 | 2.00 ± 0.18 |
| | | 0.3 | 1.68 ± 0.32 | 1.67 ± 0.21 |
| | | 0.5 | 2.66 ± 0.41 | 1.66 ± 0.23 |
| | 0.1 | 0.1 | 3.00 ± 0.41 | 2.01 ± 0.17 |
| | | 0.3 | 2.33 ± 0.21 | 3.00 ± 0.09 |
| | | 0.5 | 2.01 ± 0.08 | 3.00 ± 0.14 |
| 0.2 | 0.01 | 0.1 | 2.00 ± 0.13 | 1.66 ± 0.08 |
| | | 0.3 | 3.00 ± 0.09 | 1.068 ± 0.37 |
| | | 0.5 | 1.33 ± 0.15 | 3.00 ± 0.14 |
| | 0.05 | 0.1 | 1.66 ± 0.13 | 1.64 ± 0.18 |
| | | 0.3 | 1.00 ± 0.13 | 1.62 ± 0.15 |
| | | 0.5 | 1.00 ± 0.18 | 2.66 ± 0.24 |
| | 0.1 | 0.1 | 1.33 ± 0.22 | 1.61 ± 0.12 |
| | | 0.3 | 2.00 ± 0.43 | 1.66 ± 0.16 |
| | | 0.5 | 2.33 ± 0.11 | 2.00 ± 0.28 |

Table 2: Effect of varying conc. Of $MgSO_4.7H_2O$, $CuSO_4$, $7H_2O$, $KH_2PO_4$, on the antibacterial activity against Streptococcus pyogenes

| Conc. of $MgSO_4.7H_2O$ (%) | Conc. of $CuSO_4.7H_2O$ (%) | Conc. of $KH_2PO_4$ (%) | 48 hours incubation | 72 hours incubation |
|---|---|---|---|---|
| 0.005 | 0.01 | 0.1 | 0.33 ± 0.47 | 1.66 ± 0.23 |
| | | 0.3 | 2.00 ± 0.13 | 2.64 ± 0.43 |
| | | 0.5 | 2.33 ± 0.21 | 2.00 ± 0.14 |
| | 0.05 | 0.1 | 1.66 ± 0.37 | 1.60 ± 0.38 |
| | | 0.3 | 1.68 ± 0.41 | 2.00 ± 0.29 |
| | | 0.5 | 1.62 ± 0.32 | 5.00 ± 0.18 |
| | 0.1 | 0.1 | 0.33 ± 0.13 | 4.00 ± 0.09 |
| | | 0.3 | 0.33 ± 0.11 | 3.66 ± 0.32 |
| | | 0.5 | 1.66 ± 0.32 | 2.63 ± 0.16 |
| 0.05 | 0.01 | 0.1 | 1.63 ± 0.42 | 1.00 ± 0.44 |
| | | 0.3 | 0.31 ± 0.19 | 0.66 ± 0.82 |
| | | 0.5 | 0.33 ± 0.22 | 0.31 ± 0.73 |
| | 0.05 | 0.1 | 0.42 ± 0.14 | 0.24 ± 0.17 |
| | | 0.3 | 1.60 ± 0.38 | 0.65 ± 0.26 |
| | | 0.5 | 1.39 ± 0.42 | 0.71 ± 0.23 |
| | 0.1 | 0.1 | 0.53 ± 0.30 | 1.33 ± 0.38 |
| | | 0.3 | 1.66 ± 0.52 | 0.15 ± 0.09 |
| | | 0.5 | 1.33 ± 0.33 | 0.23 ± 0.09 |
| 0.2 | 0.1 | 0.1 | 3.00 ± 0.69 | 0.25 ± 0.21 |
| | | 0.3 | 1.23 ± 0.53 | 0.22 ± 0.07 |
| | | 0.5 | 1.60 ± 0.38 | 1.33 ± 0.19 |
| | 0.05 | 0.1 | 2.33 ± 0.27 | 2.00 ± 0.14 |
| | | 0.3 | 3.66 ± 0.15 | 0.15 ± 0.03 |
| | | 0.5 | 3.00 ± 0.39 | 0.52 ± 0.05 |
| | 0.1 | 0.1 | 1.62 ± 0.25 | 2.00 ± 0.39 |
| | | 0.3 | 1.58 ± 0.31 | 1.66 ± 0.19 |
| | | 0.5 | 0.68 ± 0.37 | 0.33 ± 0.09 |

**A: Antibacterial Activity:** The results of antimicrobial potentials of the fermented extracts at different levels of salts (in combination as well as alone) against *E. coli* and *Streptococcus pyogenes* are shown in Table 1 and 2.

**Individual effects of salts**

**$MgSO_4.7H_2O$:** Maximum antibacterial activity of the fermented broth extract was marked against 0.05% $MgSO_4.7H_2O$ where by the parameter decreased significantly against other two levels (0.005 and 0.2%) for *E. coli* at 37EC (pH 7.2) under semi shake conditions. These results were in accordance with those of Hirschhorn *et al*. (1984) who obtained maximum production of subtenolin from *B. subtilis* using 0.05% $MgSO_4.7H_2O$ in growth medium during fermentation. For *S. pyogenes* the maximal antibacterial activity was marked against 0.2%

$MgSO_4.7H_2O$ (2.074 units) after 48 hrs and against 0.005% of $MgSO_4.7H_2O$ (2.815 units) after 72 hours of incubation.

**$CuSO_4.7H_2O$:** Higher significant ($p<0.01$) effect of $CuSO_4.7H_2O$ was noted on the antibacterial activity. For *E. coil* maximum antibacterial activity (2.148 units) was noted against 0.01% $CuSO_4.7H_2O$ after 48 hours and 2.407 units against 0.1% of the salt after 72 hours.
For *S. pyogenes* the maximum activity was observed against 0.05% (1.889 units) and 0.1% (1.707 units) $CuSO_4.7H_2O$ after 48 and 72 hours, respectively. Waning antibacterial activity was noted against other concentrations. However, Johnson *et al.* (1984) reported that 0.001% $CuSO_4.7H_2O$ in the fermentation medium facilitated higher production of bacitracin from *B. subtilis*. The difference may be attributed to the difference in whole salt concentration.

**$KH_2PO_4$:** For *E. coil* the maximum antibacterial activity was observed at 0.1% $KH_2PO_4$, whereas the activity gradually decreased at the other two levels after 48 hours. These results are invigorated by findings of Landy *et al.* (1984) attempting production of bacillomycin utilizing 0.1% $KH_2PO_4$.
Against *S. pyogenes* maximum antibacterial activity (1.519 units) was recorded in the medium containing 0.1% $KH_2PO_4$ after 72 hours incubation at 37EC (pH 7.2) under semi shake conditions. While the activity was found to be lower after 48 hours at all the three $KH_2PO_4$ of concentrations. Gause *et al.* (1944) found maximum antibacterial activity of Gramicidin S from *B. brevis* using 0.2% $KH_2PO_4$ (pH 7) at 37EC (for 72 hours) incubation. The little difference observed in the results may be due to different species of the same genus and different substrate.

**b. Double Combination Effect**

**$MgSO_4.7H_2O/CuSO_4.7H_2O$:** Highly significant ($p<0.01$) effect of the combined salt conc. of $MgSO_4$ and $CuSO_4$ was found on the antibacterial activity. After 48 hours maximum antibacterial activity against *E. coil* due to combined effect of the salts, was found at 0.5/0.05% and 0.5/0.1% conc. of $MgSO_4.7H_2O/CuSO_4.7H_2O$. The maximum activity against *S. pyogenes* was found at 0.2/0.5% combination. While after 72 hrs. incubation the maximum antibacterial activity was obtained at 0.005/0.1% salt combination against both microorganisms. When the combined salt concentration of the medium was varied from its optimal level, the antibacterial activity was noted to decrease.

**$NgSO_4.7H_2O/KH_2PO_4$:** For $Mg50_4.7H_2O/KH_2PO_4$ after 48 hours the maximum anti *E. coli* activity was seen at 0.05/0.1% combination but the results were statistically non-significant ($p>0.05$). But there was a significant effect of the combined salt combination after 72 hours incubation, at which the maximal anti *E. coli* activity was found at 0.05/0.3 and 0.2/0.5% combination of $MgSO_4.7H_2O/KH_2PO_4$. Whereas the maximum anti *S. pyogenes* activity for this combination was noted at 0.2/0.1 and 0.005/0.5% after 48 and 72 hours incubation respectively. The antibacterial activity of the fermented extract was decreased significantly at other $MgSO_4.7H_2O/KH_2PO_4$ combinations.

**$CuSO_4.7H_2O/KH_2PO_4$:** Simultaneous effect of $CuSO_47H_2O/KH_2PO_4$ has revealed that maximum anti *E. coil* activity at 48 hours incubation was obtained from the medium containing 0.01% $CuSO_4.7H_2O$ and 0.3% $KH_2PO_4$. On the other hand the other combined combinations had not as good effects after 48 hours against *E. coll.* Further the combined effect of the salts had no significant effect on the anti *E. coli* activity during delayed incubation (72 hours).
Against *S. pyogenes* the maximum antibacterial activity was found at 0.05/0.3 and 0.1/0.1% of the $CuSO_4.7H_2O/KH_2PO_4$ combinations after 48 and 72 hours incubation period respectively.

**c. Triple Combination:** Maximum anti *E. coil* activity obtained after 48 hours was 3.0 units by using $MgSO_4.7H_2O/CuSO_4.7H_2O/KH_2PO_4$ combinations with following concentration respectively: 0.05/0.05/0.1, 0.05/0.1/0.1 and 0.2/0.01/0.3%. Similarly, after 72 hours the maximum antibacterial activity was same (3.0 units) but the relative concentration of $MgSO_4.7H_2O$, $CuSO_4.7H_2O$ and $KH_2PO_4$ was different (Table 1). Other combinations had lower antibacterial activity.
The maximum anti *S. pyogenes* activity was also same (3.0 units) after 48 hours of the fermented broth extract at different triple combinations of the salts. However, after 72 hours incubation the maximal anti *S. pyogenes* activity was much higher (5.0 units) by using the salt combination in concentration of 0.005, 0.05 and 0.5% $MgSO_4.7H_2O$, $CuSO_4.7H_2O$ and $KH_2PO_4$ respectively. Other two combinations also had higher antibacterial activity against *S. pyogenes* i.e. 4.0 and 3.66 units. All other combinations had lower anti *S. pyogenes* activity and had no significant effect on the antibacterial activity.
Conclusively, referring to the antimicrobial activity monitored against *E. coli* and *S. pyogenes* all the salts exhibited commendable results, particularly in delayed span of time (72 hrs) and could justifiably be taken as more appropriate for optimal yield of antimicrobial agents through fermentation of molasses by *B. subtilis*.

**B: Antifungai Activity:** *Bacillus subtilis* produce antifungal antibiotics such as iturin (Phae and Shoda, 1991; Ohno *et al.*, 1992) and bacillomycin D (Tenoux *et al.,* 1991). In the present studies, it was observed (through spore germination test) that all the extracts with variable concentrations of $MgSO_4.7H_2O$, $CuSO_4.7H_2O$ and $KH_2PO_4$ showed equally good antifungal activity when tested against *Fusarium oxysporum.* Similar finding was observed by Filippi *et al.* (1989) and Besson and Michell (1987) who reported that *Bacillus subtilis* inhibited the growth of *Fusarium oxysporum.* It was indicated that the extract which was obtained from the fermented broth completely inhibited the germination of the spores. Lima and Claudia (1990) also noted the inhibitory effect of *Bacillus subtilis* on germination and growth of *Fusarium equiseti.*

**C: Haemolytic Activity:** Extracts of all the replications did not show haemolytic activity. Apart from lytic action on bacteria, some antibiotics have haemolytic activity which is considered to be the negative property of antibiotics, So

the negative results for heamolytic activity in the present study suggest that the antibiotics produced, although crude, were of good quality.

On the basis of above discussion, it may be concluded that *Bacillus subtilis* can successfully be used to produce antibiotics by using molasses as substrate at the salt concentrations disucssed above. Further studies may be conducted to isolate and purify the antibiotics produced. It can help to solve the problem of environmental pollution caused by the agro-industrial wastes.

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