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# Optimization of Culture Conditions for Enhanced Antibacterial Activity by a Fish Gut Bacterium *Bacillus* Sp. Bsa1

Karthick.M[12]., P. Maheshkumar[3], D. Krishnamoorthy[4]., A. Prakasham[4]., M. Balachandar[5] and R.Azhagu Raj*[12]
[1]Department of Animal Science, Manonmaniam Sundaranar University, Tirunelveli, Tamilnadu, India - 627 012
*[12]Department of Zoology, St. Xavier's College, (Autonomous)
Palyamkottai, Tamilnadu India -627 002,
[3]Department of Zoology, Annamalai University, Chidambaram,
Tamilnadu, India 608002
[4]Department of Physics, Thiruvalluvar Government Arts College, Rasipuram, Namakkal - 637 401, Tamilnadu, India
[5]Department of Zoology, Loyola College, Chennai, Tamilnadu, India -600 034

**Abstract:- The present study was undertaken to investigate the antibacterial potential of a bacterium BSA1 isolated from the gut of common carp *Cyprinus carpio* against human bacterial pathogens. Polyphasic test results revealed that the candidate bacterium BSA1 belongs to the genera *Bacillus* sp. Effect of different production media on antibacterial activity inferred that Bacillus sp. grown in YBPD medium had pronounced antibacterial activity (2 and 8 mm) than other media tested. Effect of various media components on antibacterial activity substantiated that mannitol (3 to 14), peptone (2 to 10 mm), 3% inoculum (3 to 10 mm) and 40°C (2 to 6 mm) had effectively enhanced the growth of bacterium with appreciable inhibitory zones. Statistical analysis of antibacterial activity through ANOVA suggested that inhibitory zones significantly varied due to different between bacterial strains and media components. Further studies on the effect of pH, vitamins, surfactants, NaCl (%) and a thorough statistical media optimization protocol may yield suitable media for growth and bioactive metabolite by Bacillus sp.**

***Keywords:-*** *Bacillus sp.; Media optimization; Antibacterial activity.*

## I. INTRODUCTION

Microbial communities, known as microbiota, play a large role in maintaining host health through increasing digestion efficiency and use of nutrients, boosting the immune system, and preventing attachment and proliferation of opportunistic pathogens (Perez *et al.*, 2010). Fishes are in association with microbes present in the aquatic environment, and they receive bacteria from the aquatic environment through water and food. Being rich in nutrients, fish gut confers a favorable niche for microorganisms, which in turn is generally colonized by large number of heterotrophic bacteria, and also it have complex microbial ecosystem.

It is well known that, besides skin, gills of aquatic organisms are directly exposed to the environment and is colonized by great number of heterotrophic bacteria (Perez *et al.*, 2010). Interest in manipulation of the micro biota to take advantage of these benefits and to prevent disease in aquaculture has increased dramatically (Sihag and Sharma, 2012; Verscheure*et al.*, 2000). However in many fish species, the composition of the natural micro biota has not been characterized and as a result, the dominant bacterial players and their downstream influence on fish health are unclear.

In aquatic organisms, the mucosal surfaces are the primary protective barriers and harbor both beneficial and opportunistic pathogenic microorganisms (Gomez et al., 2013). Environmental conditions are known to alter the micro biota of fish (Yoshimizhu and Kimura, 1976). However, the skin-associated micro biota of fish has not been thoroughly investigated, and the interrelationship of the micro biota with the environment is not well known. (Nair 2000) reported that gill and gut is the ideal site for microbial growth, often the total viable bacterial count seems to be lesser than skin but more or less similar to intestine. (Rudresh et al. 2010) studied the microbial gill flora of a freshwater fish Garramullya (skyes) and their preliminary exploration revealed nineteen bacterial strains showing wide diversity of enzyme production, morphological and biochemical characteristics. Similarly, (Yaghobi et al. (2014) examined the intestinal micro biota of striped cat fish Pangasianodon hypophthalmus fed on dietary nucleotide after ten weeks of experimentation. Results showed progressive increase in intestinal bacterial load (CFU/g) with gram negative bacteria as the predominant groups. (Dutta et al. (2015) isolated seventeen potent bacterial isolates from the gastrointestinal tract of freshwater fish Catlacatla with extracellular enzyme (amylase, protease, lipase, cellulase, phytase and xylanase) producing ability and antagonistic activity against selected fish pathogens such as Aeromonas salmonicida, A. hydrophila and A. veronii.

Fish-microbe interactions are closely related to overall fish health. Innate immune response and nutrient metabolism depend on microorganisms that colonize the gastrointestinal tract. Therefore it is important to understand the microbial communities colonizing fish skin, gill and gut, which may help to understand the status of fish health. Thus considering the importance of fish-microbe interaction, the

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present study was undertaken to determine the antibacterial potentials of a bacterium BSA1 isolated from the gut of common carp fish *Cyprinus carpio.*

## II. MATERIALS AND METHODS

### *A. Collection and maintenance of fish gut bacterial isolate and bacterial pathogens*

In the present study a promising bacterium BSA 1 isolated from the gut of common carp Cyprinus carpio and clinical bacterial pathogens such as Escherchia coli, Enterobacter feacalis, Proteus mirabilis, Pseudomonas fluorescens, Serratia maracescens, Streptococcus mutans, Streptococcus pneumonia, Vibrio vulnificus, V. alginolyticus and V. parahaemolyticus were collected from Department of Biotechnology, Sri Kaliswari college, Sivakasi, Tamilnadu, India. The collected bacterium was stored in nutrient agar slants at 4°C for further study.

### *B. Antimicrobial activity*

Antibacterial activity of culture free supernatant (CFS) of BSA 1 was determined by agar well diffusion method. Briefly, BSA 1 was grown in tryptone broth for 24h and on the same instance overnight cultures of test pathogenic bacterial strains were also prepared. Then overnight culture of bacterial pathogen was aseptically spreaded over Muller Hinton Agar (MHA) plates using sterile cotton swabs. Thereafter wells of 6 mm diameter were punched over MHA plates using a sterile gel puncher and filled with 100 µl of CFS of BSA 1. The plates were then incubated at 37°C for 24h. The growth inhibitory activity in terms of zone of inhibition (mm) formed around each well was measured and recorded. The assay was carried out in triplicates.

### *C. Identification of candidate bacterial strain*

From the key results of primary screening (agar well diffusion method) the prominent result yielding bacterium BSA1 was subjected to identification through morphological, physiological and various biochemical characteristics. Based on the results, the candidate bacterial strain BSA 1 was identified as *Bacillus* sp.

- *Selection of suitable medium for BSA 1*

In order to find out the suitable medium for BSA1 to exhibit inhibitory activity, the candidate bacterial strain was enriched in tryptone broth. For this tryptone broth was prepared and sterilized at 121°C for 15 min and then inoculated with a loopful of culture. The flask was then incubated for 24h at 37°C. Then 2% of seed culture was transferred to various test media such as M1 →YBPD broth (Yeast extract 0.15%, Beef extract – 0.5%, Peptone 0.5%,→Dextrose – 2%, Magnesium sulphate – 0.01%, and Sodium acetate – 0.05%), M2 →MRS broth (de Man Rogosa sharpe broth), M3→Nutrient broth (Starch –0.4%, Yeast extract – 0.2% and Peptone 0.3%) and M4 →ZMB (Zobell marine broth) individually and then incubated for 24h in a rotatory shaker (120 rpm) at 37°C. Then the culture free supernatants obtained were tested against bacterial strains through agar well diffusion method.

### *D. Effect of different carbon sources on antibacterial activity*

Based on the screening results of different media on antibacterial activity by *Bacillus* sp., the most predominant result rendering production medium YBPD broth was selected for further optimization study. To achieve the maximum inhibitory activity, the production medium was individually substituted with various carbon sources such as Mannitol, Glucose, Fructose, Maltose, Inositol, Sucrose and Dextrose at 2% concentration. YBPD broth without addition of carbon source alone was used as control and incubated at 37°C for 24h in rotatory shaker (120rpm). Then 100µl of culture free supernatant was tested against previously mentioned bacterial strains through agar well diffusion method. Growth inhibitory activity in terms of zone of inhibition (mm) was measured over control was recorded.

### *E. Effect of different nitrogen sources on antibacterial activity*

To attain the highest bactericidal activity, the YBPD broth was individually substituted with various organic nitrogen sources such as Meat extract, Yeast extract, Peptone, Malt extract, Skim milk powder, Casein enzyme hydrolysate and Soybean meal. The medium without any nitrogen source was taken as control. The whole set up was incubated in rotatory shaker (120rpm) for 24h at 37°C. After 72h of incubation, 100µl of culture free supernatant (CFS) of all the experimental media were individually taken and tested against the bacterial strains through agar well diffusion method and the inhibition zone observed against the bacterial strains were noticed.

### *F. Effect of different inoculum size (%) on antibacterial activity*

In order to test the effect of different inoculum size (%) on the antibacterial activity the candidate bacterial strain was inoculated in nutrient broth at varying level of inoculum percentage such as 1, 2, 3, 4 and 5%. Then the whole set up was kept in the rotatory shaker at 120rpm for 24h at 37ºC. After the course of incubation period, 100µl of culture free supernatant from each experimental medium was taken and tested against the selected bacterial strains through agar well diffusion method and the bactericidal activity in terms of zone of inhibition (mm) was measured.

### *G. Effect of different temperature (°C) on antibacterial activity*

The effect of temperature on antibacterial efficacy in terms of zone of inhibition activity was determined by incubating the culture at different temperature such as 20, 30, 40, 50 and 60°C for 24h in YBPD broth medium. After the specified period of incubation (24h), 100µl of culture free supernatant from each sample was added to wells of MHA plates already swabbed with the bacterial strains and the inhibitory zone (mm) obtained against the test strains were recorded.

### *H. Statistical analysis*

The data obtained in the present study were subjected to the following statistical analysis using computer software Statistica 6.0 (Statosoft, UK) and EPA (Probit analysis software).

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## III. RESULTS AND DISCUSSION

*A. Primary screening of fish gut bacterial isolates for its inhibitory activity through cross – streak method*

Based on the morphological characteristics, altogether eighteen different bacterial isolates were isolated from the common carp *C. carpio*. All the isolates were individually subjected to primary screening through cross streak method against bacterial pathogens. Among the eighteen isolates screened the most predominant result rendering candidate bacterium BSA1was selected for further study.

*B. Identification of candidate bacterial strain BSA 1*

The candidate bacterium BSA 1 was subjected to polyphasic tests and the results inferred that the potent fish gut isolateBSA1belongs to the genera *Bacillus* sp. Morphological test showed that the colony was milky white coloured, regular, spreading flat, rod shaped cell morphology, gram positive and motile in nature. This bacterial strain gave positive results f or MR, citrate, catalase, oxidase and negative for indole, urease, VP and was able to hydrolyze casein and starch. Considering the sugar utilization pattern, it was found to be positive to glucose, fructose, xylose, mannose, mannitol, sorbitol, sucrose, maltose, galactose and negative towards utilizing arabinose, inositol and lactose. It grew over a wide range of pH (5–10) and temperature (30–50 °C).

*B. Selection of suitable medium for antibacterial activity*

To select a suitable medium for the candidate bacterial strain *Bacillus* sp., it was cultured individually in various media such as Nutrient broth, YBPD broth, MRS broth, ZMB and tested for maximum its antagonistic efficiency. Amongst different media tested for antagonistic activity, the candidate bacterial strain cultivated in YBPD medium (M1) exhibited maximum bioactivity activity against the bacterial pathogens by forming zone of inhibition ranged between 2 and 8 mm. Followed by this, the bacterium *Bacillus* sp. grown in MRS broth (M2) and nutrient broth(M3) media have also shown growth inhibitory activity, however bacterium grown in M2 and M3 medium showed lesser level of zone of inhibition than *Bacillus* sp. grown in YBPD broth medium(Fig1.).

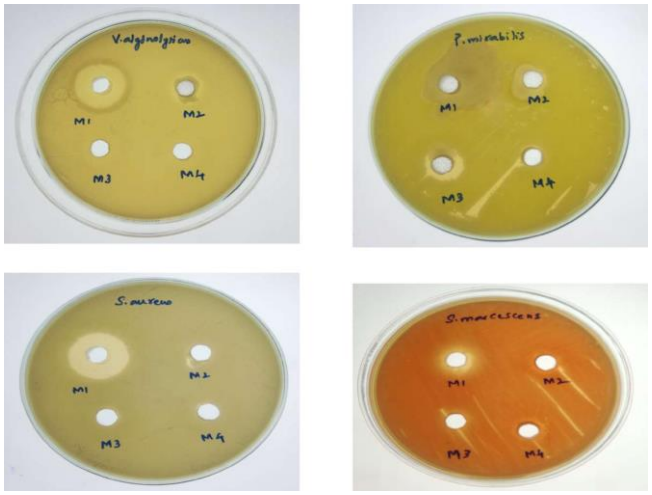

Fig 1:- Effect of different media on antibacterial activity by fish gut bacterial isolate *Bacillus* sp.

M1 – YBPD broth; M2 – MRS broth; M3 – Nutrient broth; M4 – Zobell marine broth

Here the zone of inhibition recorded was between 2 to 4 and 2 to 5mm, respectively. On the same instance, the candidate strain cultivated in zobell marine broth (M4) displayed very poor antagonistic activity. The statistical two -way ANOVA for the data on antibacterial activity as a function of variation between different test media was statistically significant ($F = 10.12$; $P < 0.05$); whereas variation between bacterial pathogens was statistically non-significant ($F = 1.5333$; $P > 0.05$).

| Source of variations | Sum of Squares | df | Mean Square | F | P-value |
|---|---|---|---|---|---|
| Variation due to bacterial pathogens | 43.125 | | 4.791667 | 1.533333 | P > 0.05** |
| Variation due to different media | 94.875 | 3 | 31.625 | 10.12 | P < 0.05* |
| Error variance | 84.375 | 27 | 3.125 | -- | - |
| Total variance | 222.375 | 39 | - | - | - |

Table 1. Two-way ANOVA for the data on antibacterial activity as a function of variation due to different bacterial strains Vs various medium

*Statistically significant ** Statistically non-significant

*C. Effect of culture conditions on antibacterial activity by Bacillus sp.*

In order to test the effect of carbon sources on bioactivity, the most predominant result rendered YBPD broth medium was selected and supplemented with individual carbon sources such as mannitol, glucose, fructose, maltose, inositol, sucrose and dextroseat 2% concentration. Medium without any of the above carbon source was considered as negative control.

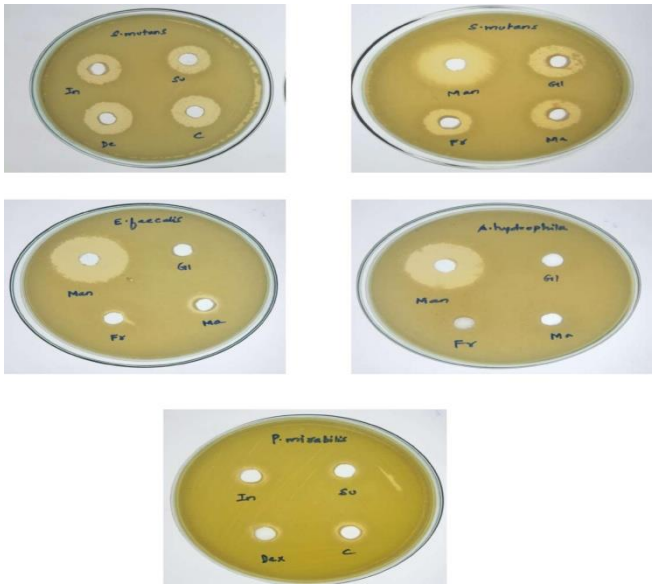

Fig 2:- Effect of different carbon sources on antibacterial activity by fish gut bacterial isolate *Bacillus* sp.

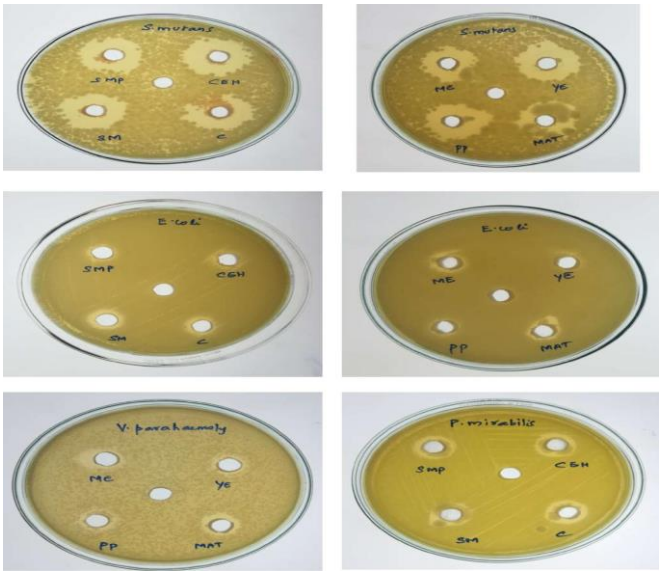

Fig 3:- Effect of different nitrogen sources on antibacterial activity by fish gut bacterial isolate *Bacillus* sp.

From the result it was observed that, media supplemented with mannitol had 100% growth inhibitory activity against all the tested bacterial pathogens by forming zone of inhibition within the range between 3 ± 0.09 and 14 ± 0.89 mm, which is comparatively higher than the zone of inhibition recorded in other carbon sources supplemented media as well as medium without carbon source (negative control) with 2 ± 0.10 to 7 ± 0.62 mm growth inhibitory activity (Fig2.). The two – way ANOVA for the data on antibacterial activity as a function of variation between carbon sources as well as variation between bacterial pathogens was statically significant ($F = 8.789956$; $P < 0.05$ and $F = 4.30138$; $P < 0.05$).

*D. Effect of different nitrogen sources*

The result on experimental medium (YBPD broth) was supplemented with various organic nitrogen sources at 0.5% concentration and tested for its significant antibacterial activity against the bacterial pathogen. Among various organic nitrogen sources tested, medium supplemented with peptone had better antibacterial activity against 80% of the tested bacterial pathogens and it ranged from 2 ± 0.10 to 10 ± 0.51 mm. Next to peptone, medium supplemented with meat extract, malt extract, skim milk powder and casein enzyme hydrolysate showed 70% growth inhibitory activity by forming zone of inhibition from 3 to 9 mm, 2 to 10 mm, 2 to 10 mm and 3 to 9 mm respectively(Fig.3.).

However yeast extract and soybean meal supplemented medium showed marginally lesser antagonistic activity. Nutrient broth medium (control) showed considerably lesser zones than peptone and other nitrogen sources supplemented medium which was ranged between 2 ± 0.10 to 9 ± 0.87 mm. The statistical two - way ANOVA for the data on antibacterial activity as a function of variation between nitrogen sources was statistically non-significant ($F$ 1.141721; $P > 0.05$); whereas variation between bacterial pathogens was statistically significant ($F = 23.85517$; $P < 0.001$).

*E. Effect of different inoculum size (%)*

Results on different size of inoculum of *Bacillus* sp. on antibacterial activity inferred that the increase in inoculum size upto a particular range showed a consistent increase in growth inhibitory activity. For instance, medium inoculated with 1 and 2% inoculum size exhibited zone of inhibition from 3 ± 0.10 to 10 ± 0.77 and 4 ± 0.13 to 16 ± 0.65mm, respectively. On the same instance, medium inoculated with 3% inoculum had extended the bioactivity, which was ranged between 2 ± 0.10 and 12 ± 0.74 mm. Amongst the tested pathogenic bacterial strains, *Streptococcus mutans* and *Proteus mirabilis* bacterial strains were found to be more sensitive.

However, further increase in inoculum size of 4 and 5% showed a gradual depletion in growth inhibitory activity against pathogenic bacterial strains which was ranged between 2 and 11 mm respectively. The statistical two-way ANOVA for the data on antibacterial activity as a function of variation between inoculum size was statistically non-significant ($F = 1.458063$; $P > 0.01$); whereas variation between pathogenic bacterial pathogens were statistically more significant ($F =$ and $49.73243$; $P < 0.001$).

*F. Effect of different media temperature*

The influence of temperature was assessed in experimental YBPD broth at different temperature such as

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20, 30, 40, 50 and 60°C. Among these, the candidate bacterial strain cultivated at 40°C inferred 100% inhibitory activity against all the tested bacterial strains with the antagonistic activity was ranged between 2 ± 0.11 and 7 ± 0.60 mm respectively. Besides, bacteria grown at 30°C recorded 2 ± 0.09 to 6 ± 0.24 mm. Further increase or decrease in temperature has shown reduction in growth inhibitory activity against pathogenic bacterial strains. For instance, the candidate bacterial strain grown at 50ºC had shown growth inhibitory activity only a trace level of activity against *Streptococcus mutans*; but it did not show bioactivity against other bacterial strains (Fig4.).

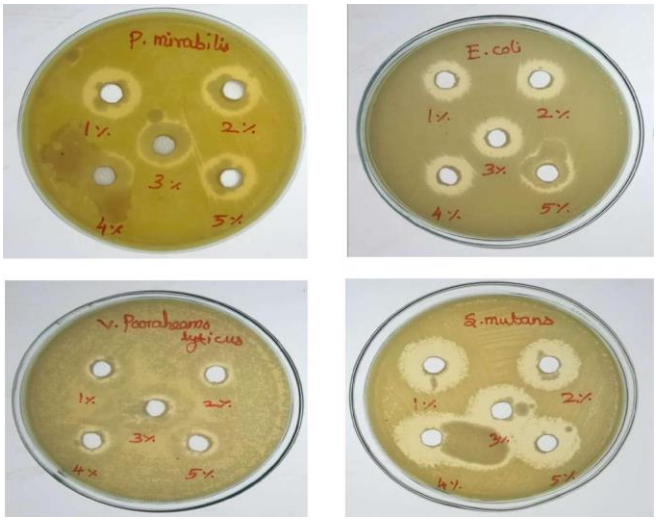


Fig 4:- Effect of different inoculum (%) on antibacterial activity by fish gut bacterial isolate *Bacillus* sp.

The candidate bacterial strain *Bacillus* sp., grown at 20 and 60°C did not grow well and showed no positive influence on growth inhibitory activity. The statistical two – way ANOVA revealed that the antibacterial activity as a function of variation between temperatures was statistically more significant ($F = 55.53079$; $P < 0.0001$); whereas variation due to different pathogenic bacterial pathogens was not statistically significant ($F = 0.985337$; $P > 0.05$).

Thus the overall results of the present study inferred that, the candidate strain grew almost well when medium was supplemented mannitol as carbon source. In accordance to the present study (Todorova and Kozhuharova (2010) evidenced that *Bacillus subtilis* isolated from soil has effectively assimilated mannitol as carbon source and displayed significant bioactivity against the both gram positive and gram negative bacterial strains. (Thakur *et al.* (2009) inferred that mannitol was found to be the best source for growth and metabolite production by Streptomyces sp. against fungus *R.* solani. In general supplementary carbon sources are important nutrient because they give sufficient energy for microbial growth and fermentation.

In order to investigate the effect of nitrogen source on antibacterial activity, the medium was substituted individually with 0.5% of different organic nitrogen sources. Results inferred that higher level of zone of inhibition in the medium supplemented with organic nitrogen sources in the order of Peptone >Meat extract>Malt extract > Skim milk powder > Casein enzyme hydrolysate. This result supports the previous findings of Oskay (2011) where he evidenced that, *Streptomyces* sp. KGG 32 an actinobacterium grew well when medium was supplemented with organic nitrogen sources such as meat extract, peptone and yeast extract. Results obtained from the present study clearly pointed out that the candidate bacterial strain requires supplementation of organic nitrogen sources for its bioactive compound production.

Inoculum plays an important role in scaling up of bioactive metabolites during fermentation process. In the present study, effect of different size of inoculum of *Bacillus* sp. on antibacterial activity displayed significant variation in the zone of inhibition with respect to inoculum size. Further increase in percentage of inoculum beyond 3% size of inoculum in production medium (4 and 5%) depicted a gradual reduction in growth inhibitory activity.

In the present study, lower growth inhibitory recorded against pathogenic bacterial strains at 1 and 2% size of inoculum indicate that concentration of inoculum is insufficient for growth and production of antimicrobial substance. However higher zone of inhibition recorded in 3% size of inoculum may be due to optimum growth and biosynthesis of bioactive metabolite. In accordance to the present study (El-sersy and Abou-Elela (2006) reported that increase in size of inoculum had a negative impact on production of bioactive metabolite and bioactivity. In general size of inoculum varies with respect to bacterium and needs to be standardized appropriately for the better growth and production of antimicrobial compound.

From the result it was noticed that *Bacillus* sp. incubated at 40°C had grew well and inferred varying levels of growth inhibitory activity against all the tested pathogenic bacterial strains (100%). This was in consistent with the results of Asha Devi *et al.* (2008), where they reported that the culture supernatant of bacterium *B. clausii* MB9 grown at 45°C has attributed 95% growth inhibitory activity (19 out of 20 strains) against the pathogenic bacterial strains ranged between 10 to 16mm, respectively.

## IV. CONCLUSION

The gut bacterial isolate BSA1 was subjected to morphological, physiological, biochemical characteristics and based on the results it was identified as *Bacillus* sp. Screening of various media for antibacterial activity by *Bacillus* sp. inferred that, bacteria grew well in YBPD medium and had pronounced growth inhibitory ranged between 2 to 8 mm against the tested pathogenic bacterial strains; whereas *Bacillus* sp. grown in other media showed a marked reduction in growth inhibitory activity. Effect of different carbon sources on antibacterial activity inferred that medium supplemented with mannitol had 100% antibacterial activity against the test bacterial pathogens with the zone of inhibition ranged from 3 ± 0.09 to 14 ± 0.89 mm.

The other tested carbon sources showed marginal reduction in antagonistic activity than mannitol substituted medium. Similarly, medium without carbon source had also showed lesser bioactivity. Effect of different nitrogen sources on antibacterial activity pointed out that the candidate bacterium *Bacillus* sp. requires peptone as organic nitrogen source. Nevertheless the inhibitory zones recorded in the other organic nitrogen sources were in the following order: Meat extract>Malt extract > Skim milk powder > Casein enzyme hydrolysate. Effect of different inoculum size (%) of *Bacillus* sp. evidenced that medium with 3% inoculum size attributed better level of growth and bioactive metabolite synthesis. Effect of different temperature on antibacterial activity by *Bacillus* sp. revealed that this bacterium grew well at 40°C and had better level of zone of inhibition of 2 ± 0.11 to 7 ± 0.60 mm against the test bacterial pathogens. Further increase or decrease in temperature did not favour growth of bacterium and thereby displayed a negative impact in bioactivity.

## V. ACKNOWLEDGMENT

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