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POTENTIAL ASSESSMENT OF BACILLUS COAGULANS AND BACILLUS CLAUSII FOR BIOREMEDIATION OF CHLORPYRIFOS AND ATRAZINE, RESPECTIVELY: AN IN VITRO STUDY

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ABSTRACT

Pesticides have been used continually for decades now. Despite strict government regulations they enter the human body via food and water where they cause various health issues ranging from weakened immune system to respiratory disorders to cancer. Probiotic microorganisms have been known to reduce the toxicity caused by these pesticides.In this study two probiotic species of genus *Bacillus* i.e. *B. clausii* and *B. coagulans* were tested to determine their ability to tolerate atrazine and chlorpyrifos, respectively. It was found that the minimum inhibitory concentration (MIC) of *B. coagulans* was not obtained as it grew till 9% of chlorpyrifoswhereby *B. clausii* had an MIC of 2% for atrazine. Even at such high concentration they did not show anychange in their morphological and biochemical characteristics after growth on atrazine and chlorpyrifos, respectively. When tested for change in its antibiotic sensitivity it was found that upon exposure to chlorpyrifos the sensitivity of *B. coagulans* decreased for ciprofloxacin but increased for ampicillin, norfloxacin and chloramphenicol and remained constant for kanamycin. In case of *B. clausii* sensitivity decreased for norfloxacin and ciprofloxacin whereas the sensitivity increased for ampicillin, chloramphenicol, kanamycin and gentamycin. Both the probiotic species, post exposure to their respective pesticides, did not exhibit any change in their acid and bile tolerance. This leads to the conclusion that the microorganisms studied have the ability to tolerate pesticides much above the permissible limits and may have the potential for bioremediation of pesticides in *in vivo*.

Keywords: *atrazine, chlorpyrifos, bioremediation, B. clausii, B. coagulans, probiotics.*

I. INTRODUCTION

India is amongst the top ten countries of the world in pesticide consumption. This has led to increase in yield of crops and has improved the financial status of farmers and hence the country's economy. Unfortunately, most of the pesticide residue contaminate soil and water and have managed to enter the food chain. From here they have entered the human body and are causing deleterious effects ranging from weakened immune system to respiratory disorders to cancer (Blair et al., 1990, Baker and Wilkinson, 1990, Kamel and Hoppin, 2004, Ritter et al., 2006). These pesticides can be herbicides, rodenticides or fungicides and are classified on the basis of their structure i.e. they include organochlorine, organophosphorus, carbamates and nitrogen based pesticides (Vaccari et al., 2006). Many conventional technologies have been tried to eliminate them from the environment like landfills, recycling and pyrolysis but such methods have led to formation of toxic intermediates, thus compounding the problem (Paul et al., 2005). Other disadvantages include high cost of these procedures and difficulty of execution (Jain et al., 2005). Microorganisms are eco-friendly, cost effective and have the ability to survive in the presence of toxic pesticides. Hence, bioremediation became the method of choice. Many studies have reported the capability of different genera of microorganisms for bioremediation of pesticides. These genera include *Flavobacterium, Arthobacter, Aztobacter, Burkholderia, Bacillus* and *Pseudomonas* (Glazer and Nikaido, 2007). Some of the species of the genus *Bacillus* have probiotic properties and so theymight have the potential for bioremediation of pesticides thereby reducing their toxicity in *in vivo.*

II. MATERIAL AND METHODS

The organisms for the study, *Bacillus coagulans* and *Bacillus clausii*, were procured in the form of commercially available tablet, Sporlac-DS and as spore suspension with the name Enterogermina,respectively from the local

chemist. They are Gram positive motile rods and arefacultatively anaerobic. *B. coagulans* was studied for its tolerance against chlorpyrifos while *B. clausii*was tested against atrazine. Both are commonly used pesticidesand were obtained from the local market.

Chlorpyrifos is a broad spectrum insecticide. It is used to kill root worms, fleas, flies, beetles. Along with corn, cotton, peaches, apples and grains it is used as an insecticide for lawns and ornamental plants. It is a contact poison as well as stomach poison (Mallicket al., 1999). In humans, it shows neurological effects, persistent developmental disorders and autoimmune disorders. Atrazine is a herbicide used to inhibit unwanted broad leaf plants in agriculture. (Siripattanakul et al., 2009). It is specially used on maize and sugarcane crops as well as on golf turfs and lawns. Due to its moderate persistence in soil and moderate water solubility it remains in soil and water for long durations (Wang et al., 2011, Wang and Xie, 2012). It has many effects on human health due to its properties of carcinogenicity and teratogenicity (Batra et al., 2009, Shenoy 2012). Six different antibiotics [kanamycin (30 µg), norfloxacin (10 µg), gentamycin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), and ampicillin (10 µg)] were obtained from HiMedia, India.

Determination of minimum inhibitory concentration (MIC) of *B. coagulans* and *B. clausii* after exposure to pesticides

The MIC of *B. coagulans* was tested forchlorpyrifosand for *B. clausii* againstatrazineby method of Mistry et al (2010).Plate dilutions of these pesticides were prepared in nutrient agar (NA) medium ranging from 0.5% to 9%. All the plates were inoculated with 24 hrs old test culture and incubated at 37^{o}C for 24 hrs.

The lowest concentration of the pesticide that inhibited the visible growth on the NA plate was considered as MIC for the respective pesticide.

Characterization of bacteria after exposure to chlorpyrifos and atrazine

To check whether the pesticides have caused any changes to the cultures under study, *B. coagulans*and *B. clausii*were morphologically and biochemically characterized after exposure to atrazine and chlorpyrifos respectively.

Morphological characterization

After exposure to pesticides, *B. coagulans* and *B. clausii*were characterized according to cell shape, size and arrangement and staining properties like Gram staining reaction and endospore staining.

Biochemical characterization

Catalase production

Slants of NA were inoculated with the *B. coagulans*after exposure to chlorpyrifos and *B. clausii* exposed to atrazineand incubated at 37^{o}C for 24 hrs. Two to three drops of H_2O_2 were added on the visible growth and observed for vigorous bubbling within 10s.

IMViC test

The IMViC tests consists of four different types of tests (a) Indole production test, (b) Methyl red- VogesProskauer test, (c) Citrate utilization test.

a) Indole production test

1% tryptophan broth wasinoculated with test organisms after they had been exposed to their respective pesticides and incubated at 37° C for 48 hours. 1 ml of Kovac's reagent was added to the broth and immediately observed for appearance of layer of cherry red colour on top of the broth.

b) Methyl Red-VogesProskauer (MRVP) test

To determine glucose fermentation products, MRVP test was performed in glucose phosphate (GP) broth tubes. Two tubes of GP broth each were inoculated with chlorpyrifos exposed *B. coagulans* and atrazine exposed *B. clausii*while one uninoculated tube was kept as control. All the tubes were incubated for 72-96 hrs at 37^{o}C. For MR test 5 drops of methyl red indicator was added and observed for development of red colour immediately. For VP test 12 drops of VP reagent I and 2-3 drops of VP reagent II were added and the broth

was exposed to air by removing the caps and mixed intermittently and observed for half an hour for colour change from pink to crimson.

c) Citrate utilization test

Simmon's citrate agar slants were inoculated with test organisms after they had been exposed to their respective pesticides and incubated at 37° C. After 48 hrs, slants were observed for change in colour from green to blue.

Antibiotic sensitivity

This test was performed using unexposed *B. coagulans* and *B. clausii*as well as test organisms after exposure to pesticides to observe whether the pesticide exposure has caused any changes in their sensitivity towards antibiotics. For this both unexposed and exposed cultures of each organism were tested for six different antibiotics mentioned above by Kirby-Bauer disc diffusion method (Bauer et al., 1966). The inoculated plates were incubated at 37^{o}C for 24hrs and results were compared on the basis of their zone of inhibition.

Characterization for probiotic efficacy

Bile tolerance

The bile tolerance of the exposed cultures was estimated by the methodology of Hassanzadazar et al., 2012with some modifications. The cultures were exposed to their respective pesticides in NB at 37^{o}C overnight. A saturated bile solution was prepared which was used as stock. Three working concentrations (0.20, 0.30 and 0.40%) were prepared in nutrient broth while 0% served as control sample. The cultures were incubated at 37^{o}C. Viable number of bacteria were enumerated by making a 10-fold serial dilution in 0.10% peptone water every hour for 3hrs and plating on nutrient agar plates and incubating for 24 hrs at 37^{o}C. The hourly sample was also monitored spectrophotometrically at 600nm. The experiments were done in triplicates.

Acid tolerance

The cultures were grown overnight at 37^{o}C in NB with pesticide. To test tolerance to acidic pH, the methodology of Hassanzadazar et al. (2012) was followed with some modifications. 1% of each exposed culture was added to nutrient broth of pH 2, 3, and 4. The cultures were incubated at 37^{o}C.Viable number of bacteria were enumerated by making a 10-fold serial dilution in 0.1% peptone water every hour for 3hrs and plating on nutrient agar plates and incubating for 24 hrs at 37^{o}C. The hourly sample was also monitored spectrophotometrically at 600nm. The experiments were done in triplicates.

III. RESULTS AND DISCUSSION

Minimum inhibitory concentration (MIC)

Many studies performed on environmental strains of *Bacillus* genus have shown tolerance to chlorpyrifos (Liu et al., 2012, El-Helow 2013, Ishag et al., 2016) and atrazine (El- Bestawy et al., 2013, Wang et al., 2014, Balakrishnan and Athilakshmi,2016). In present study, the MIC of *B. clausii*for atrazine was found to be 2% while *B. coagulans* exhibited growth till 9% of chlorpyrifos, hence no MIC was obtained.This indicates that both the probiotic species might have some potential in bioremediation of respectivepesticides.

Chlorpyrifos exposed B. coagulans

After growth in the presence of chlorpyrifos,*B. coagulans* was characterized morphologically and biochemically, the results of which are shown in Table 1.

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Table. 1: Comparison of morphological and biochemical characteristics of B. coagulans and B. clausiibefore and after exposure to chlorpyrifos and atrazine respectively.

S. No.	Characteristics	Unexposed *B. coagulans* and *B. clausii*	Exposed *B. Coagulans* and *B. clausii*
1.	**Colony characteristics**	Creamish, opaque, small	Creamish, opaque, small
2.	**Gram's staining**	Gram positive	Gram positive
3.	**Endospore staining**	Endospore present	Endospore present
4.	**Catalase test**	Positive	Positive
5.	**Indole production**	Positive	Positive
6.	**MR test**	Positive	Positive
7.	**VP test**	Negative	Negative
8.	**Citrate utilization**	Positive	Positive

Both unexposed and exposed cultures showed resistance against various antibiotics. The results showed that the sensitivity decreased for ciprofloxacin (26mm to 8 mm) and gentamycin (29 mm to 27 mm) whereas the sensitivity increased for ampicillin (9 mm to 18 mm), norfloxacin (17 mm to 32 mm), kanamycin (27 mm to 30 mm) and chloramphenicol (18 mm to 28 mm)upon exposure to chlorpyrifos.

Table. 2: Diameter of zone of inhibition (mm) of B. coagulans before and after exposure tochlorpyrifosfor different antibiotics.

Name of Antibiotic	Unexposed *B. coagulans*	Exposed *B. coagulans*
Ciprofloxacin	26	08
Chloramphenicol	18	28
Gentamycin	29	27
Ampicillin	09	18
Norfloxacin	17	32
Kanamycin	27	30

As the culture under study is probiotic in nature, *B. coagulans* was evaluated for its probiotic efficacy after exposure to chlorpyrifos by checking for its bile tolerance and acid tolerance. This culture showed maximum optical density signifying highest bile tolerance at 0.30% bile (w/v) after 3 hrs (Fig 1). As the OD was very high, a confluent growth was observed and individual colonies could not be counted.

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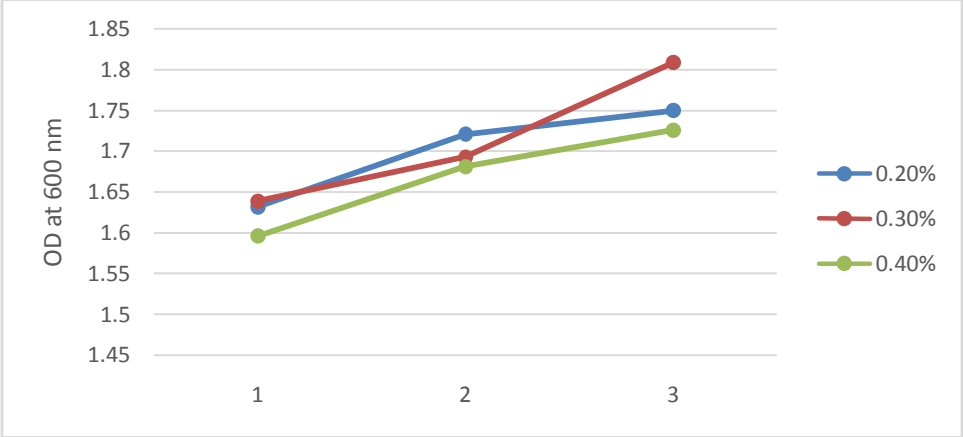

Fig. 1: Bile tolerance of B. coagulans after exposure to chlorpyrifos (OD at 600 nm)

For acid tolerance, the maximum OD was observed at pH 3 after 3 hrs (Fig 2). As was the case with bile tolerance, colonies could not be counted due to dense growth of the culture.

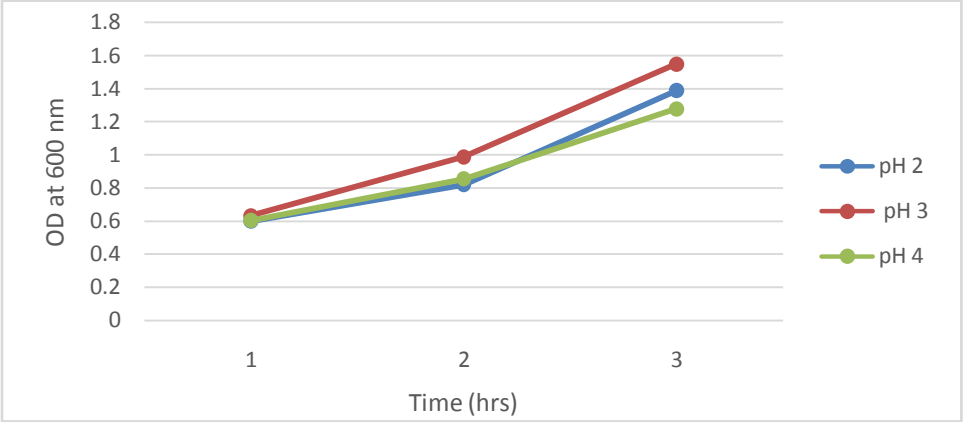

Fig. 2: Acid tolerance of B. coagulans after exposure to chlorpyrifos (OD at 600 nm)

B. clausiiafter exposure to atrazine

Upon evaluation of the morphological and biochemical characters of *B. clausii* post exposure to atrazine, it was observed that it did not show any change in its colony morphology, staining properties and biochemical characters indicating that it did not undergo any change in presence of pesticide (Table 1). When the culture was tested for its antibiotic sensitivity for various antibiotics listed above, the result showed that the sensitivity decreased for norfloxacin (23 mm to 20 mm) and ciprofloxacin (24 mm to 18 mm) whereas the sensitivity increased for ampicillin (12 mm to 19 mm), chloramphenicol (10 mm to 15 mm),gentamycin (22 mm to 25 mm) and kanamycin (22 mm to 26 mm) (Table 3).

Table. 3: Diameter of zone of inhibition (mm) of atrazine exposed B. clausii for different antibiotics.

Name of Antibiotic	Unexposed *B. clausii*	Exposed *B. clausii*
Ciprofloxacin	24	18
Chloramphenicol	10	15
Gentamycin	22	25
Ampicillin	12	19
Norfloxacin	23	20
Kanamycin	22	26

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When *B. clausii* was tested for its bile tolerance it was observed that it showed maximum absorbance and maximum number of colonies at 0.30% bile after 3 hrs (Fig 3 and 4).

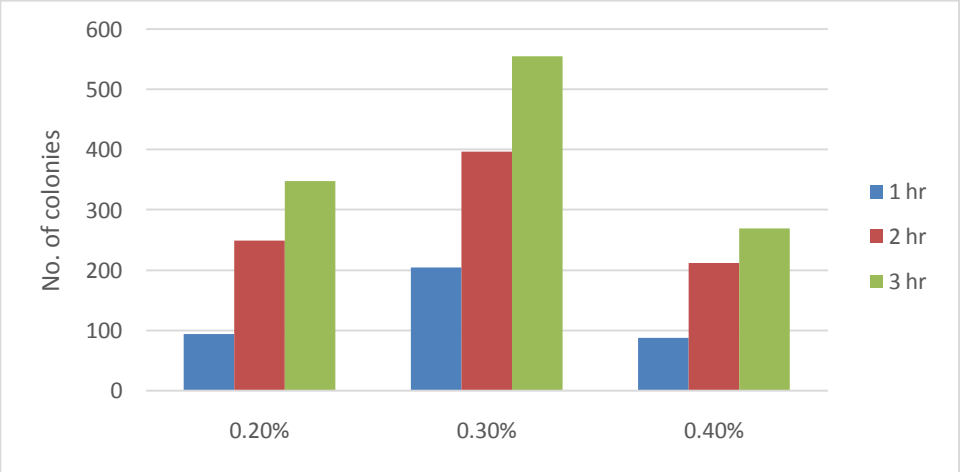

Fig. 3: Bile tolerance of B. clausiiafter exposure to atrazine (number of colonies)

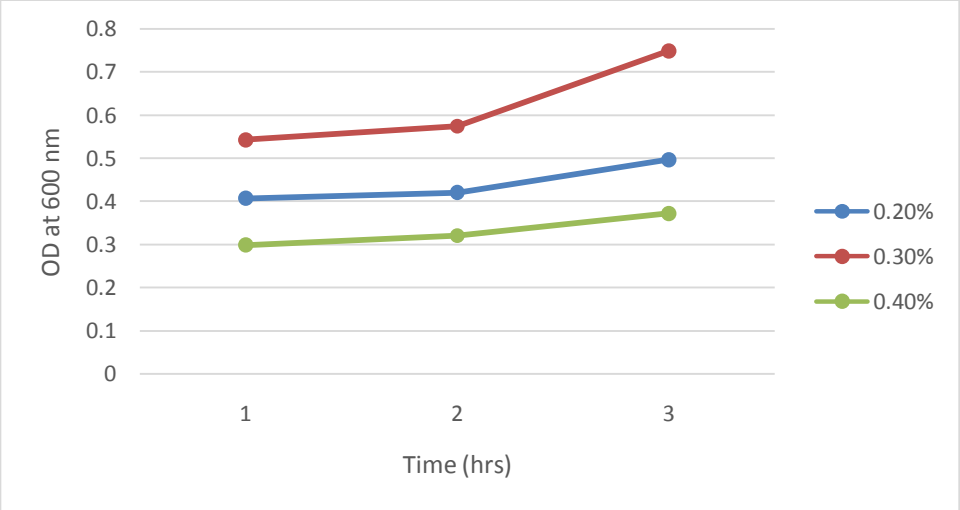

Fig. 4: Bile tolerance of B. clausiiafter exposure to atrazine (OD at 600 nm)

Similarly, it showed no change in its acid tolerance i.e. maximum number of colonies and maximum OD was recorded at pH 3 after 3 hrs (Fig 5 and 6).

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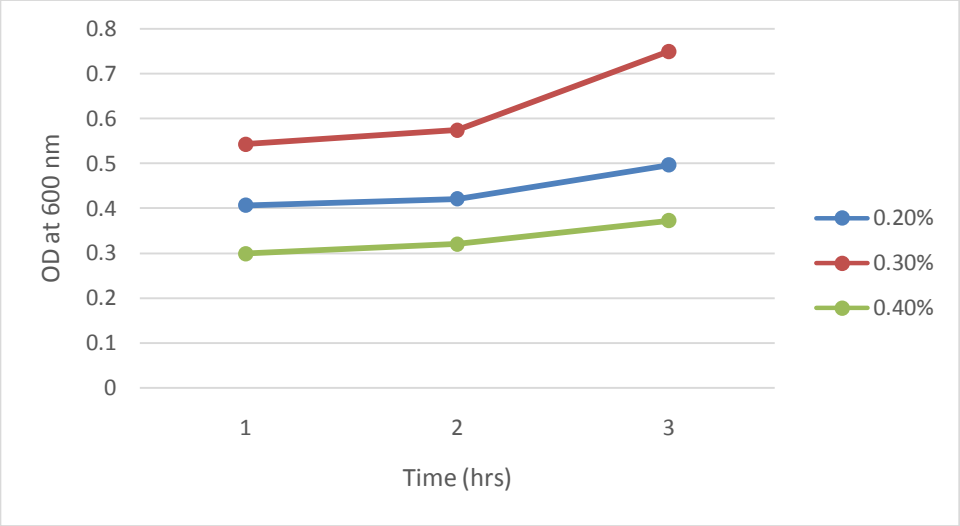

Fig. 5: Acid tolerance of B. clausiiafter exposure to atrazine (number of colonies)

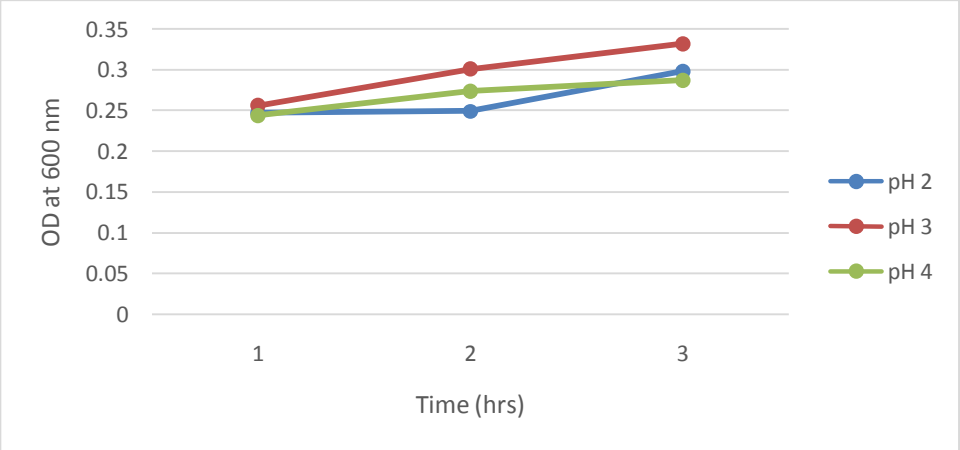

Fig. 6: Acid tolerance of B. clausii after exposure to atrazine (OD at 600 nm)

Pesticides are known mutagens (Chauhan and Singhal, 2006) whose exposure can cause change in characteristics of the organism. However, in the present study both the organisms did not undergo any change in their morphological and biochemical characteristics. Any probiotic bacteria should have the ability to survive, grow and function in the gut even when it encounters varying conditions (Hyronimus et al., 2012). It should be able to tolerate gastric pH ranging from 2.5-3.5 (Holzapfel et al., 1998) at the same time it should grow well in presence of bile salt concentration ranging from while 0.20%- 2.0% (Gunn, 2000). A probiotic should survive at a critical bile concentration of 0.30%, (Gilliland et al., 1984, Zhou et al., 2007, Hyronimus et al., 2012) while the pH standardized for probiotic is 3 (Sahadeva et al., 2011). Both *B. coagulans* and *B. clausii* under study are already established commercially available probiotics which did not lose their probiotic efficacy even in the presence of 9% and 1% chlorpyrifos and atrazine stress respectively, indicating that they both have the potential to be used in pesticide bioremediation *in vivo*.

IV. CONCLUSION

The study undertaken has proved that the commercially available probiotics *B. coagulans* and *B. clausii*can tolerate chlorpyrifos and atrazine respectively. Their MIC indicates that, they have the potential for bioremediation of the

respectively tested pesticides. The cultures did not change even after exposure to high concentrations of pesticides. Moreover, the pesticide stress did not affect their probiotic efficacy as well. Hence, it is concluded that *B. coagulans* and *B. clausii* have the potential for gut bioremediation of the respectively tested pesticides. However, further studies are needed to prove their role *in vivo*.

REFERENCES

[1] *Baker, S. (1990). The effect of pesticides on human health. Advances in modern environmental toxicology.*
[2] *Balakrishnan, L., &Athilakshmi, N. (2016). Degradation of atrazine by Pseudomonas sppand Bacillus spp from Saccharumofficinarum(sugar cane) fields of southern India and its potential application in bioremediation. Asian journal of science and technology,7(5), 2903-2911.*
[3] *Batra, M., Pandey, J., Suri, C. R., & Jain, R. K. (2009). Isolation and characterization of an atrazine-degrading Rhodococcus sp. strain MB-P1 from contaminated soil. Letters in applied microbiology, 49(6), 721-729.*
[4] *Bauer, A. W., Kirby, W. M., Sherris, J. C., &Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. American journal of clinical pathology, 45(4), 493.*
[5] *Blair, A., Axelson, O., Franklin, C., Paynter, O. E., Pearce, N., Stevenson, D. & Woods, J. (1990). Carcinogenic effects of pesticides. Advances in modern environmental toxicology (USA).*
[6] *Chauhan, R. S., &Singhal, L. (2006). Harmful effects of pesticides and their control through cowpathy. International Journal of Cow Science, 2(1), 61-70.*
[7] *El-Bestawy, E., Sabir, J., Mansy, A. H., &Zabermawi, N. (2013). Isolation, identification and acclimatization of Atrazine-resistant soil bacteria. Annals of Agricultural Sciences, 58(2), 119-130.*
[8] *El-Helow, E. R., Badawy, M. E., Mabrouk, M. E., Mohamed, E. A., & El-Beshlawy, Y. M. (2013). Biodegradation of chlorpyrifos by a newly isolated Bacillus subtilis strain, Y242. Bioremediation journal, 17(2), 113-123.*
[9] *Gilliland, S. E., Staley, T. E., & Bush, L. J. (1984). Importance of Bile Tolerance of Lactobacillus acidophilus Used as a Dietary Adjunct1. Journal of dairy science, 67(12), 3045-3051.*
[10] *Glazer, A. N., &Nikaido, H. (2007). Microbial biotechnology: fundamentals of applied microbiology. Cambridge University Press.*
[11] *Gunn, J. S. (2000). Mechanisms of bacterial resistance and response to bile. Microbes and infection, 2(8), 907-913.*
[12] *Hassanzadazar, H., Ehsani, A., Mardani, K., &Hesari, J. (2012). Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. In Veterinary Research Forum (Vol. 3, No. 3, p. 181). Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.*
[13] *Holzapfel, W. H., Haberer, P., Snel, J., & Schillinger, U. (1998). Overview of gut flora and probiotics. International journal of food microbiology, 41(2), 85-101.*
[14] *Hyronimus, B., Le Marrec, C., Sassi, A. H., &Deschamps, A. (2000). Acid and bile tolerance of spore-forming lactic acid bacteria. International journal of food microbiology, 61(2-3), 193-197.*
[15] *Ishag, A. E. S., Abdelbagi, A. O., Hammad, A. M., Elsheikh, E. A., Elsaid, O. E., Hur, J. H., & Laing, M. D. (2016). Biodegradation of chlorpyrifos, malathion, and dimethoate by three strains of bacteria isolated from pesticide-polluted soils in Sudan. Journal of agricultural and food chemistry, 64(45), 8491-8498.*
[16] *Jain, R. K., Kapur, M., Labana, S., Lal, B., Sarma, P. M., Bhattacharya, D., & Thakur, I. S. (2005). Microbial diversity: application of microorganisms for the biodegradation of xenobiotics. Current science, 101-112.*
[17] *Kamel, F., &Hoppin, J. A. (2004). Association of pesticide exposure with neurologic dysfunction and disease. Environmental health perspectives, 112(9), 950.*
[18] *Liu, Z. Y., Chen, X., Shi, Y., & Su, Z. C. (2012). Bacterial degradation of chlorpyrifos by Bacillus cereus. In Advanced Materials Research (Vol. 356, pp. 676-680). Trans Tech Publications.*
[19] *Mallick, K., Bharati, K., Banerji, A., Shakil, N. A., &Sethunathan, N. (1999). Bacterial degradation of chlorpyrifos in pure cultures and in soil. Bulletin of environmental contamination and toxicology, 62(1), 48-54.*

[20]*Mistry, K., Desai, C., Lal, S., Patel, K., & Patel, B. (2010). Hexavalent chromium reduction by Staphylococcus sp. isolated from Cr (VI) contaminated land fill. International journal of Biotechnology and biochemistry, 6(1), 117-129.*

[21]*Paul, D., Pandey, G., Pandey, J., & Jain, R. K. (2005). Accessing microbial diversity for bioremediation and environmental restoration. TRENDS in Biotechnology, 23(3), 135-142.*

[22]*Ritter, L., Goushleff, N. C. I., Arbuckle, T., Cole, D., &Raizenne, M. (2006). Addressing the linkage between exposure to pesticides and human health effects—research trends and priorities for research. Journal of Toxicology and Environmental Health, Part B, 9(6), 441-456.*

[23]*Sahadeva RPK, Leong SF, Chua KH, Tan CH, Chan HY, Tong EV, Wong SYW, Chan HK. Survival of commercial probiotic strains to pH and bile. Int Food Res J., 2011; 18(4): 1515-22.*

[24]*Shenoy, K. (2012). Environmentally realistic exposure to the herbicide atrazine alters some sexually selected traits in male guppies. PLoS One, 7(2), e30611.*

[25]*Siripattanakul, S., Wirojanagud, W., McEvoy, J., Limpiyakorn, T., & Khan, E. (2009). Atrazine degradation by stable mixed cultures enriched from agricultural soil and their characterization. Journal of applied microbiology, 106(3), 986-992.*

[26]*Vaccari, D. A., & Strom, P. F. (2006). Aleman. J E. Environmental Biology for Engineers and Scientists. John Wiley & Sons.*

[27]*Wang, J., Zhu, L., Liu, A., Ma, T., Wang, Q., Xie, H., & Zhao, R. (2011). Isolation and characterization of an Arthrobacter sp. strain HB-5 that transforms atrazine. Environmental geochemistry and health, 33(3), 259-266.*

[28]*Wang, J., Zhu, L., Wang, Q., Wang, J., &Xie, H. (2014). Isolation and characterization of atrazine mineralizing Bacillus subtilis strain HB-6. PloS one, 9(9), e107270.*

[29]*Wang, Q., &Xie, S. (2012). Isolation and characterization of a high-efficiency soil atrazine-degrading Arthrobacter sp. strain. International biodeterioration& biodegradation, 71, 61-66.*

[30]*Zhou, X. X., Pan, Y. J., Wang, Y. B., & Li, W. F. (2007). In vitro assessment of gastrointestinal viability of two photosynthetic bacteria, Rhodopseudomonaspalustris and Rhodobactersphaeroides. Journal of Zhejiang University SCIENCE B, 8(9), 686-692*