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RESEARCH ARTICLE

COMPARISION OF EFFECT ON THE POTENCY OF BACITRACIN SECRETED BY *BACILLUS PUMILUS, BACILLUS SUBTILIS AND BACILLUS LICHENIFORMIS* MUTATED BY ULTRAVIOLET RADIATION AND HEAVY METALS

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Abstract

Present study is carried out to show a comparison of the potency check of Bacitracin produced by *Bacillus pumilus, Bacillus subtilis and Bacillus licheniformis* treated with the Ultra Violet radiation and heavy metals. These microbes were isolated from the Sewage Treatment plant of MUST residential campus, Lakshmangarh, Sikar. Molecular identification of isolates was done by DNA isolation, PCR amplification using 16s rDNA primer and partial sequencing of purified product after sequencing. Sequences were submitted in NCBI with designated accession no. There is growing awareness of the need for development of new antimicrobial agents for the treatment of human, animal and plant diseases. A special fermentation media, given by Hanlon & Hodges, was used for the production of Bacitracin. *Micrococcus luteus* was used as a test organism. Zones of inhibition were observed of both wild and mutant strains. A standard Zinc Bacitracin (70 IU/ml) was taken for calculating potency of the sample Bacitracin. The potency of antibiotic Bacitracin (IU/ml) was determined by agar diffusion method given by William. Results indicated that the potency was highest of *B.subtilis* and least by *B.licheniformis*. This study is made to use native or indigenous micro flora for bioremediation which is useful and beneficial rather than commercial inoculum. *Bacillus* itself will be enough for the biodegradation of the complex molecules.

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INTRODUCTION

Bacitracin (C66H103N17016S) is metal reliant branched cyclic polypeptide molecular weight 1470 Daltons produced by *Bacillus licheniformis* and *Bacillus subtilis* (Azevedo et al., 2002) . Synthesis of Bacitracin happens to be nonribosomally by the large multienzyme complex BacABC. Bacitracin is directed primarily against Gram-positive bacteria via inhibition of cell wall (MacIver et al., 2006) . Bacitracin includes a mixture of structurally similar polypeptides from 12 amino acids (Kang et al., 2001). It is most commonly used in complex with zinc that seems to stabilize the antibiotic complex. It is less permeable through gastrointestinal tract as well as from skin and mucosal surfaces.

Bacitracin was first discovered in 1943 & named after a culture of *Bacillus* and the last name of a 7 year old American girl, Margaret Tracey, as it was from her wounds that the *Bacillus* was isolated. It is one of the most important antibiotics used in human medicine, topical application and used after surgical operations (MacIver et al., 2006) . It is widely used for topical therapy, for skin and eye infections; it is effective against Gram positive bacteria, including strains of *staphylococcus* that are resistant to penicillin.

Use of Bacitracin is also common in animal and poultry feed additives which upsurges feed effectiveness and reduces terror to infectious diseases (Hampson et al., 2002) (Prescott and Baggot, 1993). Regardless of its widespread use, resistance to bacitracin is still infrequent. It has also been noted that bacitracin has no noticeable negative impact on human health. Many scientists had upgraded the bacitracin yields of *Bacillus licheniformis* by treating its vegetative cells with UV and chemical mutagens. Vegetative cells of *Bacillus licheniformis* were treated with UV irradiation and then cultured on medium containing Fe^{+2} ions and pantothenic acid. Along with bacitracin production, mutagens were also used to enhance the production of Penicillin G, Phenyl-L Alanine and Actinomycin-D (Ikram and Ali, 2006) (Rubina et al., 2010) (Vandana et al., 2008). Some scientists treated *Bacillus licheniformis* cells with 0.5 M ethylmethanesulphonate for three hours and cultured in a medium containing soybean meal, sucrose and mineral salts. In another study vegetative cells of *Bacillus licheniformis* were exposed with N-methyl-N'-nitro-N-nitrosoguanidine by which bacitracin production was significantly increased (Liyong et al., 1988). A better yield of bacitracin was obtained by treatment of cells with MNNG and this suggested that blocking of alternate pathways of intermediates could very well increase the bacitracin production.

Due to wide spread use of bacitracin, it is necessary to find out ways and measures to reduce the cost of this product. To achieve this, our focus was to utilize appropriate strain improvement technology and optimization of fermentation processes that could minimize the product costs that allows microbial factories to yield higher titers of bacitracin.

Metals play an integral role in the life processes of microorganisms. Some metals serves as micronutrients, and are used in redox reactions, help in building the osmotic pressure e.g. cobalt, iron, magnesium, manganese, sodium, nickel, zinc, etc. (Bruins, et.al, 2000). Whereas, there are some heavy metals which do not play any useful roles in the biological processes, are non-essential and are toxic to the microorganisms like aluminum, cadmium, lead, mercury, silver, gold, etc. The heavy metals after entering inside the biological system interfere with the normal metabolism of the body and cell. Toxicity occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Bruins et. al, 2000) (Nies, 1999)**.**

Table 1 shows some of the metals and their harmful effects on metabolism.

Sewage is water-carried wastes, in either solution or suspension that is intended flow away from a community. Also known as wastewater flows, sewage is the used water supply of the community and is characterized by its volume or rate of flow, its physical condition, its chemical constituents and the bacteriological organisms that it contains. Depending on their origin, wastewater can be classed as sanitary, commercial, industrial, agricultural or surface runoff.

The spent water from residences and institutions, carrying body wastes, washing water, food preparation wastes, laundry wastes and other waste products of normal living are classed as domestic or sanitary sewage. Liquid-carried wastes from stores and service establishments serving the immediate community, termed commercial wastes are included in the sanitary or domestic sewage category if their characteristics are similar to household flows. Wastes that result from an industrial process or the production or manufacture of goods are classed as industrial wastes. Their flows and strengths are usually more varied, intense and concentrated than those of sanitary sewage. Surface runoff, also known as storm flow or overland flow, is that portion of precipitation that runs rapidly over the ground surface to a defined channel. Precipitation absorbs gases and particulates from the atmosphere, dissolves and leaches materials from vegetation and soil, suspends matter from the land, washes spills and debris from urban streets and highways and carries all these pollutants as wastes in its flow to a collection point.

Wastewater from all of these sources may carry pathogenic organisms that can transmit diseases to humans and other animals contain organic matter that can cause odour and nuisance problems hold nutrients that may cause eutrophication of receiving water bodies and can lead to ecotoxicology. Proper collection and safe nuisance-free disposal of the liquid wastes of a community are legally recognized as a necessity in an urbanized, industrialized society**.**

Raw wastewater contains significant concentration of heavy metals that are not degraded by the conventional process of wastewater treatment. The main source of heavy metals is the industrial activities such as metal processing, mining and electroplating, tanning, carpet washing and dyeing. Presence of high concentration of toxic heavy metals in wastewater directly leads to both contamination of receiving water bodies and deleterious impact on aquatic life (Moten and Rehman, 1998) . Use of such polluted water for consumption and other purposes can bring severe problems to human health. At higher concentration, heavy metals form toxic complex compounds in the cell that are too dangerous for any biological function. Among the microorganisms, bacteria, yeast and protozoa are generally the first category to be exposed to heavy metals present in the environment. Intrinsic bacteria, which are capable of metal accumulation, existing in soil on or near the site of contamination have adapting mechanisms to the

contaminant. Naturally occurring bacteria that are capable of metal accumulation have been extensively studied since it is difficult to imagine that a single bacterium could be capable to remove all heavy metals from its polluted site (Clausen, 2000). Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals. Among the various adaptation mechanisms, metal sorption, mineralization, uptake and accumulation, extracellular precipitation and enzymatic oxidation or reduction to a less toxic form and efflux of heavy metals from the cell has been reported (Mergeay, 1991) (Hughes.,1991) (Nies, 1992) (Urrutia and Beveridge, 1993) (Joshi-Tope and Francis, 1995) .

2. Materials And Methods

2.1 Site Description

The sewage treatment plant belongs to the university Mody University of Science and Technology situated in Lakshmangarh town in Sikar District of Rajasthan State in India. Lakshmangarh is located at 27.8225°N 75.025278°E. It has an average elevation of 222 meters (728 ft.). The agriculture is based on the monsoon rains. The average rainfall in the area is also very less. The sewage treatment plant has a large reservoir of sewage treatment from where the sample was collected.

2.2 Isolation Of Bacterial Strains By Plating

1gm of sewage was mixed with 10ml of sterile distilled water. An aliquot of 0.1 ml of dilutions for each sewage sample was spread onto nutrient agar plates. The isolated bacterial colonies will then be identified on the basis of morphological, cultural and biochemical characteristics following Bergey's Manual of Determinative Bacteriology.

2.3 Physiological And Biochemical Characteristics

Physiological and biochemical characteristics, including morphology, grams stain, temperature and pH range for growth, tolerance of different NaCl concentrations and resistance to antibiotics , enzymatic activity were tested. The cultures *of Bacillus licheniformis, Bacillus pumilus* and *Bacillus subtilis* isolated from sewage samples.

2.4 Inoculum Preparation

The inoculum was developed in 250 ml conical flask containing 25 ml medium having composition (g/L); peptone 10, Glucose 5, Beef extract 5, NaCl 2.5, $MnCl_2$ 0.7. The flask was incubated overnight at 37°C at 200 rpm. The 0.3 ml (6%) from the overnight culture was used to inoculate the 50 ml LB medium in 250 ml flask and incubated at 37°C for 6-7 hours in rotary shaker at speed of 250 rpm until O.D 600 reached at 1.5.

2.5 Production Of Bacitracin In Fermentation Media By Wild Strains

2.6 Shake Flask Fermentation

Twenty five milliliter culture medium contained in 250 mL conical flasks was autoclaved at 121°C for 20 minutes. The medium was then inoculated with 5% (v/v), 20 hours old vegetative inoculum of all three strains of *Bacillus* for the production of antibiotic bacitracin. The shake flask cultures were incubated at 37°C for about 48 hours on rotatory shaker at 200 rpm. The cells were removed by centrifugation at 10,000 rpm for 10 minutes. The clear supernatant was collected and used for antibiotic assay to determine bacitracin potency. All the bioassay experiments were performed in triplicate.

2.7 Ultra Violet Treatment and heavy metal treatment

Bacillus strains were sustained on nutrient agar slants. Cell suspension was prepared from 24-hour culture, centrifuged and washed with phosphate citrate buffer (pH7.5) twice. It was suspended in phosphate citrate buffer and diluted to10-4-10-6 times, transferred to a sterile petridish and exposed to UV lamp at 250 nm for different time intervals ranging between 5-45 minutes. The distance between lamp and suspension was adjusted at 6cm for each trial. After UV exposure, the cell suspension was transferred on nutrient agar plates. The plates were then incubated overnight at 37°C. Bacitracin production was investigated in comparison with control (cells without exposure). Mutant strains with high bacitracin yield developed from culture of *Bacillus licheniformis* by UV irradiation were separated and sub cultured in slants. Zinc bacitracin (70 IU/mg) was purchased from Sigma. The bacitracin standard was weighed accurately to give 45 IU/mL concentration using N/100 HCl as diluent. The stock solution was stored

at 4°C. Effect of potency of bacitracin was checked on two concentrations (0.1 µl/ml & 0.2 µl/ml)of metals in individually in the fermentation medium. After incubation of 48hrs at 37˚C their potencies were checked.

2.8 Bioassay Of Bacitracin

The potency of antibiotic bacitracin (IU/mL) was determined by agar diffusion method William (1977). LB agar medium was autoclaved and 20 mL of the medium was poured aseptically in the sterile petri plates and were allowed to congeal. In the meanwhile, sterilized LB agar medium (assay medium) having 50-60°C temperature was inoculated with predetermined concentration of *Micrococcus luteus* by using broth culture prepared by the inoculation of Micrococcus luteus. Four mL of the inoculated melted assay medium was spread uniformly over the first layer and was allowed to solidify. The plates were refrigerated at 4°C and used according to the need. At the time of assay the plates were taken out from the refrigerator and 4 wells of 0.8 cm diameter were made in each plate aseptically with sterilized stainless steel bores of uniform edge and size. The pieces of agar from the dug wells were picked and removed with the help of sterilized loop. The two opposite wells were filled with the working standard of 1:4 dilutions and marked as S1 and S2 respectively. The remaining two were filled with the sample whose potency was to be determined in the same dilution (1:4) and marked T2 and T2 respectively. One hundred and twenty micro liter bacitracin standards as well as samples were poured with the help of micropipette in the dig holes. The plates were then placed carefully (to avoid spreading of solution due to tilting of the plates) in incubator for 18-24 hours at temperature 37°C. Clear zones of inhibition were developed and diameter of zones of inhibition were measured and compared with the known standard. The potency of antibiotic Bacitracin (IU/ml) was determined by agar diffusion method given by William (1977).

2.9 Calculation of potency

The potency of the sample was calculated by the following formula:

Difference due to doses:

$E = ½ [(T2+S2) – (T1+S1)]$………………………………. (1)

Difference due to sample:

$F = ½ [(T2+T1) – (S1+S2)]$……………………………….(2)

Log ratio of doses:

$I = \log 4$

Slope:

$B = E/I$……………………………………………………(3)

$M = F/B$…………………………………………………..(4)

Potency ratio:

Antilog M

Potency of sample = Potency of standard x antilog M = X units/mL

where

S2 = Standard High (in concentration)

S1 = Standard Low (in concentration)

T2 = Test High

T1 = Test Low

3. RESULTS

3.1 Isolation Of Strains:

Bacillus pumilus, Bacillus subtilis and *Bacillus licheniformis* were isolated from sewage. The bacitracin activity of this strain was determined by measuring the zone of inhibition.

Composition Of The Medium:

A synthetic medium containing Glucose, 2.7gm; Na2HPO4.12H2O, 14.3gm2; KH2PO4, 4.5gm; NH4Cl, 0.535gm; MgSO4.7H2O, 0.13gm; MnCl2.4H2O, 0.01gm, FeSO4.7H2O, 0.003gm; CaCl2.6H2O, 0.02gm was used in this study.

3.2 Potency Of Wild Strains

Inhibition zones were measured and then the potency were calculated by the formula given above in the equation segment.

Table 1. Composition of media used for production of Bacitracin
(Fermentation Media)(Hanlon & Hodges, 1981)

Glucose	**2.7 gms**
$Na_2HPO_4.12H_2O$	**14.32 gms**
KH_2PO_4	**4.5 gms**
NH_4Cl	**.535 gms**
$MgSO_4.7H2O$	**.13 gms**

Table 2. Potency Of Wild Strains

STRAINS	**POTENCY (IU/ml)**
B.licheniformis (R2)	7.65
B.subtilis (K2)	20.65
B.pumilus (T1)	10.35

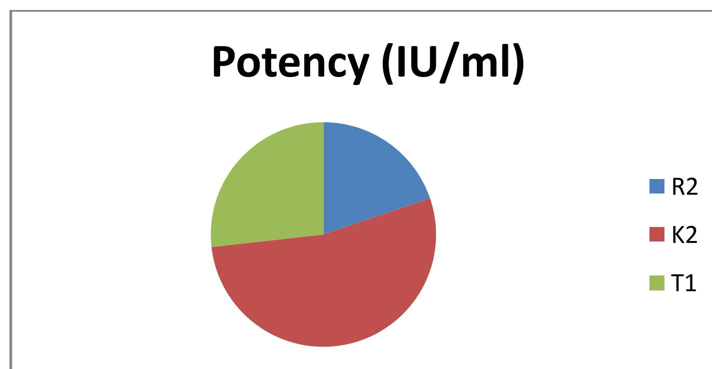

Figure 1. Pie chart distribution of potency of wild strains

3.3 Potency Of Mutant Strain

Table 3. Potency Of Mutant Strain on exposure to Ultra Violet Radiation (250 nm)

Time of UV exposure	***B.licheniformis*(IU/ml)**	***B.Subtilis*(IU/ml)**	***B.pumilus*(IU/ml)**
5 Min.	9.85	20.65	10.8
10 Min.	14.35	21.6	17.1
15 Min.	16.44	34.13	33.075
20 Min.	23.18	34.13	33.07

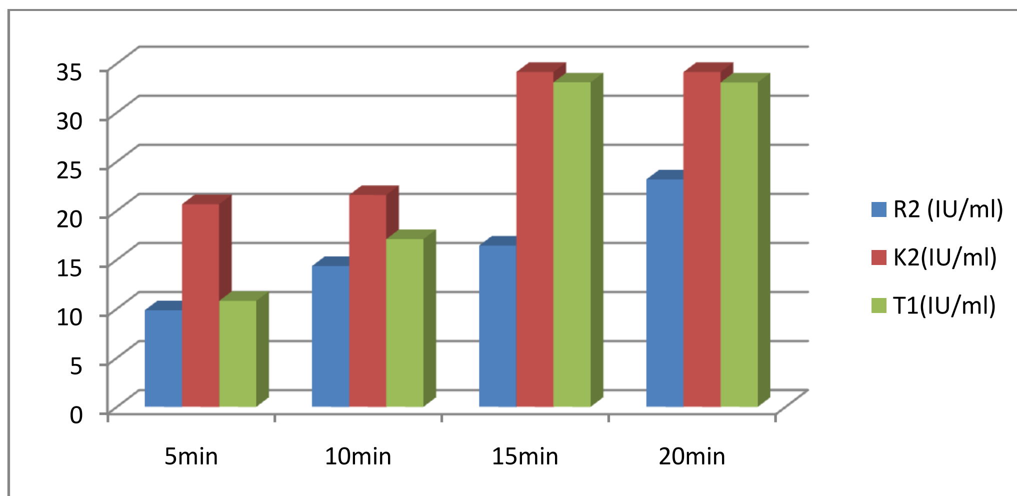

Figure 2. Graph showing bar distribution of potency after UV exposure

3.4 Effect of metals on the potency of bacitracin

Table 4. Potency of Bacitracin in the presence of metals in the fermentation media (concentration of metals 0.1 µl/ml)

METALS 0.1(µl/ml)	POTENCY (IU/ml)		
	B.licheniformis R2	*B.subtilis* K2	*B.pumilus* T1
Ni	15.96	26	31.806
Hg	7.65	14	10.35
Co	12.69	23.23	14.47
Cu	15.96	26	10.35
Zn	18.24	36.91	18.18
Mn	15.96	28.32	14.47
Mg	7.65	14.985	7.65
Fe	15.96	26	18.18
Cr	7.65	10.23	12.645

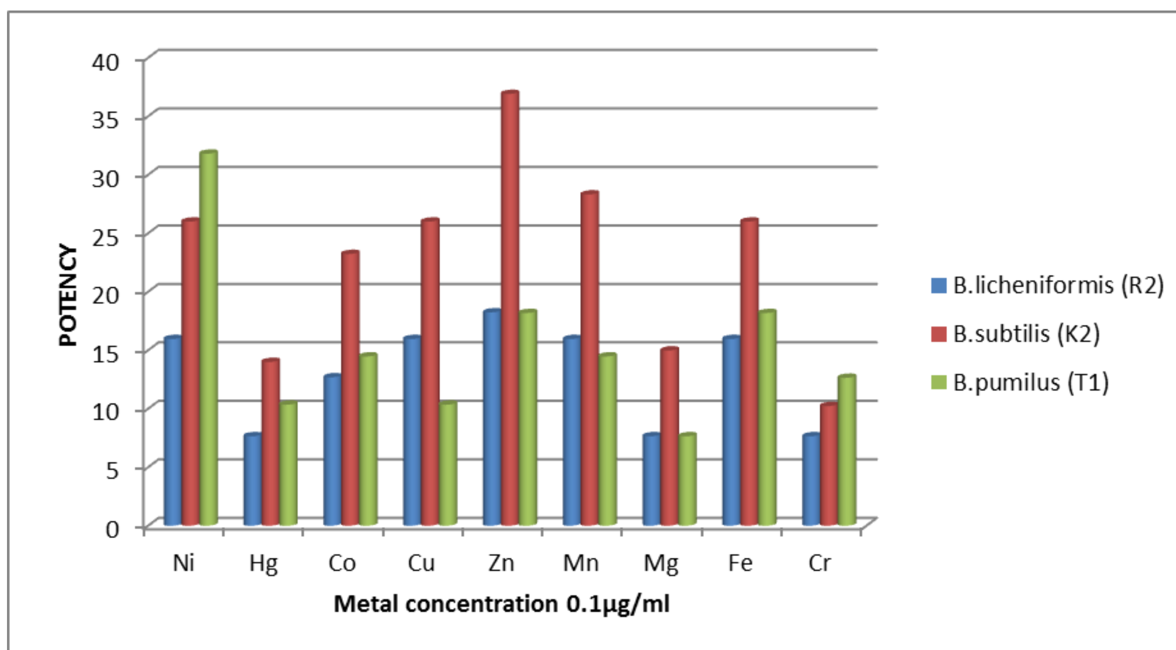

Figure 3.Graph showing the effect of heavy metals at 0.1µg/ml on the potency of Bacitracin produced by R2, K2 & T1.

Table 5. Potency of Bacitracin in the presence of metals in the fermentation media (concentration of metals 0.5 µl/ml)

METALS 0.5 µl/ml	POTENCY (IU/ml)		
	B.licheniformis R2	***B.subtilis*** K2	***B.pumilus*** T1
Ni	19.12	13.095	37.53
Hg	NIL		
Co	14.47	26	18.24
Cu	7.65	21.23	10.35
Zn	24.84	41.85	10.35
Mn	7.65	21.33	24.84
Mg	7.65	14.985	7.65
Fe	7.65	21.23	10.23
Cr	7.65	10.23	7.65

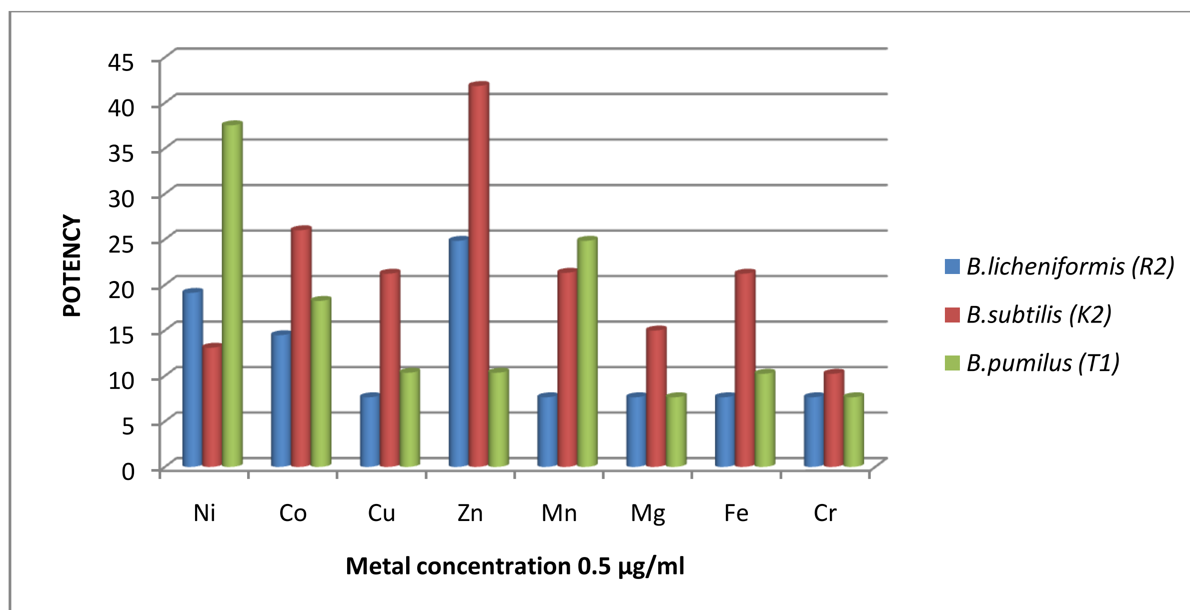

Figure 4.Graph depicting the effect of heavy metals at 0.5µg/ml on the potency of Bacitracin produced by R2, K2 & T1

3.5Comparision of potency of bacitracin produced by mutant strains and parent strains.

Table 6. Comparison of potency of bacitracin produced by mutant strains and parent strains.

	POTENCY OF BACITRACIN IN (IU/ml)					
pH	***B.licheniformis*** **R2** **uv20**	***B.Subtilis*** **K2** **uv15**	***B.pumilus*** **T1** **uv15**	***B.licheniformis*** **R2** **parent**	***B.Subtilis*** **K2** **parent**	***B.pumilus*** **T1** **parent**
5 pH	19.31	29.07	30.04	5.97	18.21	9.15
6 pH	21.06	31.42	31.73	6.32	18.92	9.23
7 pH	23.18	34.13	33.075	7.65	20.65	10.35
8 pH	20.36	32.02	32.073	7	20.05	10.04

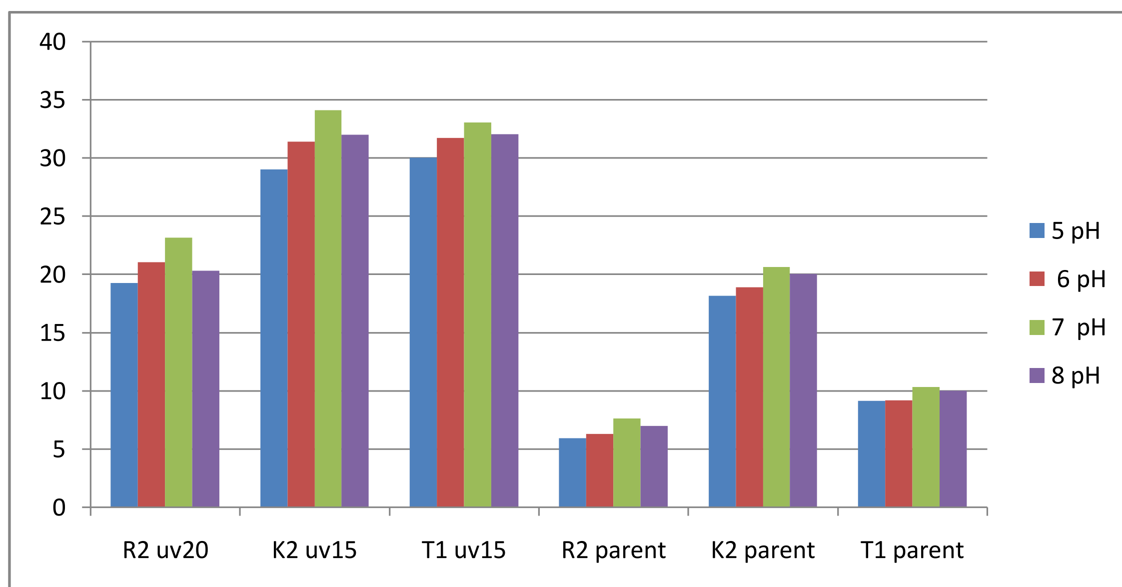

Figure 5. Graph showing the Comparison of potency of bacitracin produced by mutant strains and parent strains.

3.6 Effect of temperature on the potency of mutant and wild strains

Table 7. Comparison of potency of bacitracin produced by mutant strains and parent strains on various temperatures.

Temp-erature	*B.licheniformis* R2 uv20	*B.subtilis* K2 uv15	*B.pumilus* T1 uv15	*B.licheniformis* R2 parent	*B.Subtilis* K2 parent	*B.pumilus* T1 parent
28°C	19.02	30.86	30.21	7.01	18.78	7.81
30°C	22.35	32.18	31.01	7.03	19.04	8.57
32°C	23.02	33.05	31.57	7.65	19.5	9.02
35°C	23.06	33.78	32.81	7.65	20.02	9.05
37°C	23.18	34.13	33.075	7.65	20.65	10.35
40°C	23.03	33.97	32.08	7.5	20.01	9.9
42°C	21.9	32	30.6	7.01	19.62	8.1
45°C	21.4	29	27.8	6.3	17.91	8.03
47°C	18.2	28.05	25.4	6	15.75	6.5

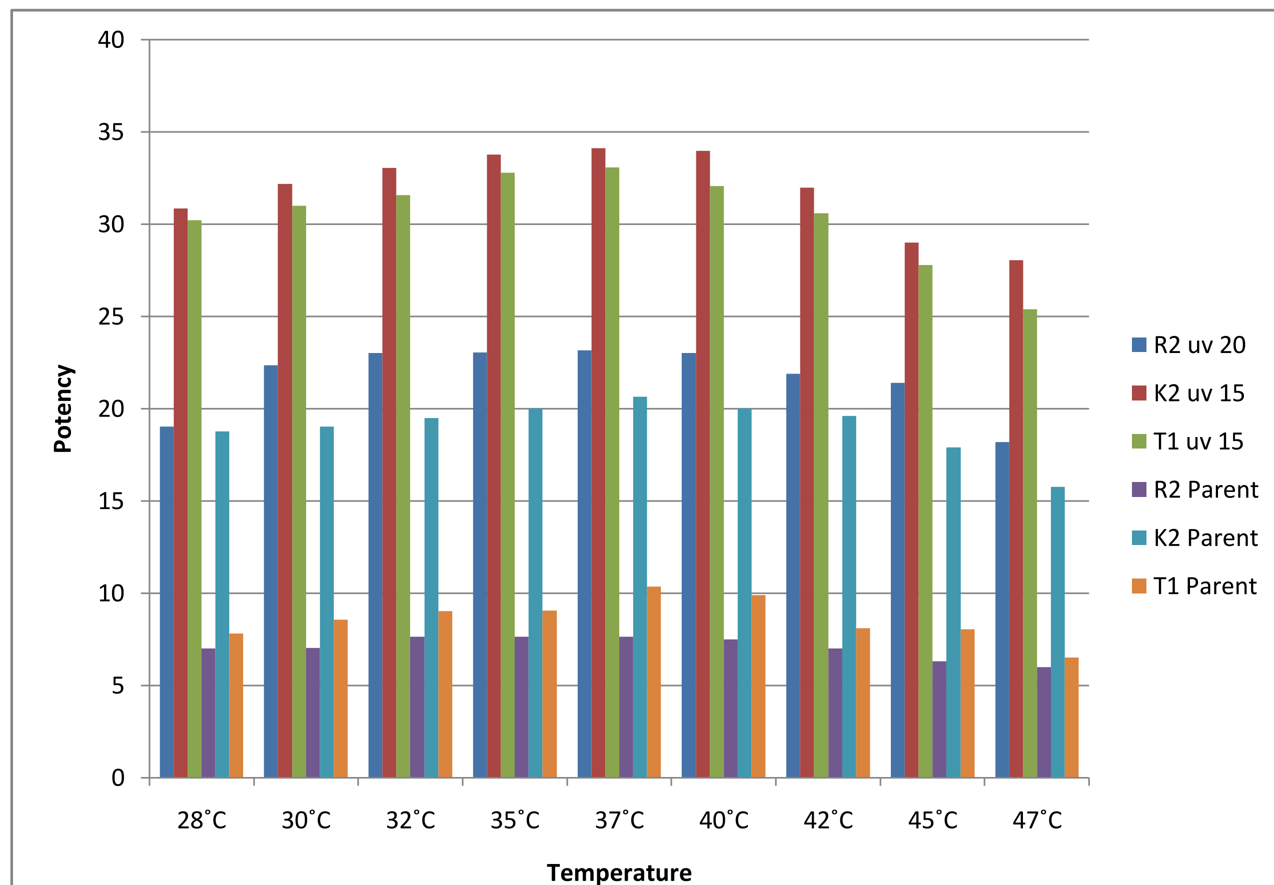

Figure 6. Graph showing the Comparison of potency of bacitracin produced by mutant strains and parent strains on different temperatures .

4. Conclusion:

When wild strains were inoculated in the fermentation media the potency observed by every individual strain was *B.licheniformis* 7.65 IU/ml, *B.subtilis* 20.65 IU/ml & *B.pumilus* 10.35 IU/ml which was drastically increased when it was treated with ultraviolet radiation (250nm) for different time intervals. Potency of Bacitracin produced by *B.licheniformis* was increased from 7.65IU/ml to 23.18IU/ml. Potency of Bacitracin produced by *B.subtilis* was increased from 20.65IU/ml to 34.13IU/ml. Potency of Bacitracin produced by *B.pumilus* was increased from 10.35IU/ml to 34.075IU/ml. Maximum level of potency of bacitracin could be seen in the time interval of 15 minutes & 20 minutes of exposure.

Effect of potency of bacitracin was checked on two concentrations (0.1 µl/ml & 0.2 µl/ml)of metals in individually in the fermentation medium. *B.licheniformis* R2 in presence of different heavy metals at 0.1 µl/ml showed a good increase in the potency of bacitracin produced by R2 accept three metals mercury, magnesium & chromium. There was no increase seen in presence of these three metals by R2. Maximum potency was given in the presence of zinc metal. B.subtilis K2 showed a decrease in presence of mercury, magnesium and chromium. Maximum potency was shown in the presence of Zinc and minimum was observed at mercury. *B.pumilus* T1 showed a decrease in the presence of magnesium. There was no change in the potency in presence of mercury. Maximum potency by T1 was given in presence of nickel.

B.licheniformis R2 in presence of different heavy metals at 0.5 µl/ml showed change only in presence of nickel, cobalt and zinc. R2 showed an increase potency on these metals. R2 showed no change in presence of copper, manganese, magnesium, iron, chromium at the high concentration of 0.5 µl/ml. maximum potency was 24.84 in presence of zinc.

B.subtilis K2 showed an increase in the potency in presence of cobalt, copper, Zinc, manganese and iron. However there was a decrease in potency in presence of nickel, magnesium and chromium. Maximum potency was observed in the presence of zinc.
B.pumilus T1 showed an increase in the potency in the presence of nickel, cobalt and manganese. It showed a decrease in presence of magnesium and chromium. Whereas there was no change in presence of copper, zinc and iron. We could not study the potency of Bacitracin of all the three *Bacillus* species in presence of mercury at 0.5 µl/ml, because there was no growth at this high concentration of mercury.

Strains which gave the highest potency after the ultra violet exposure were taken as mutant strains and compared with the parent strains for their potencies at different hydrogen ion concentrations. All the six strains showed maximum potency on pH 7. However all the six strains were able to give a good potency at acidic condition of pH 5, 6 & basic condition of pH 8. Whereas potency at acidic conditions were less than the basic condition.
All the mutant and parent strains were checked for their potencies at different temperatures at 7 pH. All strains were able to work on all the temperatures from 28˚C, 30˚C, 32˚C, 35˚C, 37˚C, 40˚C, 42˚C, 45˚C, 47˚C. all the strains gave highest potency on 37˚C. however at high temperatures the potency of all the strains was decreased .

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