ISOLATION OF BACTERIOCIN FROM LACTIC ACID BACTERIA (LAB) AND IT'S ANTIBACTERIAL ACTIVITY

Mr. Vinod Kumar Bhana

(Assistant Professor Department of Botany Bansur P.G. College Bansur Alwar-301402)

INTRODUCTION

Mankind has exploited lactic acid bacteria (LAB) for thousands of years for the production of fermented foods because of their ability to produce desirable changes in taste, flavor and texture due to the production of organic acid and hydrogen peroxide as well as to inhibit pathogenic and spoilage microorganisms to produce antimicrobial substance such as bacteriocin.

The use of nonpathogenic microorganism and their metabolite to improve microbiological safety and extend the shelf life of food. Antibiotics are at present restricted for use in food and feed, bacteriocins are an interesting group of biomolecule with antimicrobial and antifungal properties that may represent a good alternative of antibiotics. One of the concerns in food industry is the contamination by pathogens, which are frequent cause of food borne diseases. Over the past decade, recurrent outbreaks of diarrhea, combined with the natural resistance of the causative agents, contributed to its status as hazard. The problem of selection of resistant bacteria to antibiotics and the increasing demand for safe foods, with less chemical additives, has increased the interest in replacing these compounds by natural products, which do not injure the host or the environment.

Bacteriocins are ribosomaly synthesized antimicrobial peptide that has a direct inhibitory effect on harmful bacteria. Bacteriocin do not develop resistance in harmful and closely related bacteria through mutation based on scientific literature survey so far and is not – toxic, it is highly resistant to intestinal enzyme. Bacteriocin kills the microorganism rapidly by destroying or permeating the microbial membrane and impairing the ability to carry out anaerobic process. These peptides are therefore unlike to face the same antimicrobial resistance mechanism that limit current antibiotics use. It is a natural antibiotic which can potentially illicit allergic reaction in human and other medical problem.

Bacteriocins and bacteriocins producing strain of lactic acid bacteria (LAB) have been focus of extensive research in recent years due to their potential as biopreservatives. Initial studies on bacteriocins production by LAB were focused on isolated associated with dairy products. More recently bacteriocinogenic activity

has been discovered in bacterial strain from meat, silage and fermented food sour dough red wine and malting and brawling environment.

Antibiotics are generally considered to be secondary metabolite that are inhibitory substance in small concentration excluding the inhibition caused by metabolic by- products like ammonia, organic acids and hydrogen peroxide.

Material and Methods

Culture Strain-

Twenty two selected *Lactobacillus* (LAB) strain such as *Bf1, Bf2, Bf3, Bf4, Bf5, Bf6, Bf7 Bf8, Bf10, Bf14, Bf15, Bf16, Jf2, Jf4, Jf6, Jf10, Jf13, Jf14, Jf15, J1, J6, J7.* Isolated from traditional fermented food "Rabri" used in the study were obtain from stock culture of Department of Microbiology M.D.S. university Ajmer. All strain was maintained on De Mann Rogosa Sharpe (MRS) Agar and Nutrient Agar slant at -4^{o}C for further study.

Indicator Strain-

Pre isolated Strain of *E-coli 144* and *Micrococcus M3* & *M73* were used as indicator strain. And maintain on nutrient medium and MRS medium at 4^{0}C. For further study.

Isolation and Purification of Crude Bacteriocin

Lactobacillus species (LAB) were grown on 100ml MRS broth (pH7.0) for 24 hrs at 37^{0}C. For extraction on crude Bacteriocin a cell free solution were obtain by centrifuging the broth at 12000 g/rpm for 20 min at 4^{0}C. After centrifugation the supernatant were decanted and adjust the 7.0pH by means of 1N NaoH to exclude the antimicrobial activity.

Purification of Bacteriocin-

Ammonium sulphate precipitation

Purification of Bacteriocin was carried out by ammonium sulphate precipitation. The crude Bacteriocin samples produced were treated with solid ammonium sulphate to 30, 35, 40, 45, 50, 55, and 60% saturation. The mixtures were stirred for 2hrs at the 4^0C. Later centrifugation at 20,000g /rpm for 30 min at 4^0C. The precipitate were decanted and re-suspended in 25 ml of 0.05M Potassium Phosphate Buffer Solution (pH 7.0).

Dialysis was followed in a tabular cellulose membrane against 2 lit of the 0.05M Potassium phosphate buffer for 18 hrs in spectropor No.4 dialysis tubing. Assay of Bacteriocin activity was carried out and determine by agar well diffusion method. After protein precipitation the protein profiling SDS page carried out to determine the length of protein Bacteriocin.

Trichloroacetic acid (TC) Precipitation-

5% equivalent of TC was added to 25 ml of supernatant to precipitate the protein mixture was centrifuged 12000 g/ rpm for 10 min at 4^0C.after which the supernatant was decanted and the resulting of pellet was dissolved in 2ml of 0.05M potassium phosphate buffer solution and determine the molecular size of protein Bacteriocin by SDS page method and assay was carried out agar well diffusion method.

Screening of Crude Bacteriocin- (Anti bacterial Activity)

The well isolated colonies of *Lactobacillus* Bacteriocin producing strain were picked up and transferred in to 5ml MRS broth (pH 6.5) in to test tube and incubate for 24 hrs at 37^0C. After the well growth the 1ml of broth transferred in to the 100 ml of MRS broth (pH 6.5) and incubated for 24hr at 37^0C.

After incubation cell were removed by centrifugation of the culture at 12000, g/rpm for 20 min at 4^0C. Decanted the supernatant and adjust the pH (7.0) of supernatant by means of 1N NaoH to exclude the antimicrobial effect of acid. This is further known crude Bacteriocin. This crude was used for assay of antibacterial activity.

Bacteriocin assay –

Antimicrobial activity of crude Bacteriocin were performed by agar well diffusion method or assay.

Well diffusion method-

The well diffusion direct assay (Described by Schillinger and Lucke 1989: Takahiro *et al.* 1991.) procedure was used. The culture supernatant which was known as crude Bacteriocin was used for Bacteriocin assay. For this the indicator test organism E-coli 144 and M3 & M73 were grown on 10 ml Nutrient Broth for 24 hrs. 100 ml of the Nutrient Agar (NA) autoclaved and adjust the pH 7.0. After autoclaving the molten agar was temperate at 40^0C and inoculated with the indicator organism at the middle of exponential phase to a final concentration of 1%. 20-25 ml of inoculated medium was poured in sterile Petri dishes and allowed to solidify. After agar solidification, 4-5 wells of 5 mm diameter were cut with the use of borer in to each patri plate.

50µl of Crude Bacteriocin were pipetted in to each well and the plates were pre- incubated at refrigerator for 2hrs for the diffusing the crude around the wells and finally incubated directly at 37^0C for 24 hrs. Diameter of Zones produced in assay plates was measured in millimetre and image of agar palate were taken by Gel documentation camera. For all inhibition zones, the means of largest and shortest diameter was calculated. Average zone diameter are presented in this work assay experiment were done in triplicates.

Turbidometric assay

The Turbidometric assay experiment of isolated crude Bacteriocin was tested with indicator (*E-coli 144)* strain at different time interval and take optical density at 650 nm. Each 100 ml of nutrient broth flask (pH 7.0) inoculated with 5% overnight growth culture of indicator strain. After inoculation optical density was measured at 0h at 650nm.

After one hour 1% of crude Bacteriocin was added to each flask incubated the flask at 37^0C and measured turbidity by optical density at 650 nm wavelength at different time 12, 24, 36, 48, 60, 72 hrs interval. The variation in incubation time is based on the fact that the slope corresponding to the effect of the Crude Bacteriocin will vary through the time of growth. Culture flask in which the indicator stain without Crude Bacteriocin were used as controls. Curve was obtained from experimental data and the percentage of inhibition of growth of the test microorganism was estimated against the control. Assay experiments were done in triplicates.

SDS PAGE - Electrophoresis

The molecular size of the purified Bacteriocin was determined using SDS PAGE gel as described by (Sambrrok et al., 2006).

Preparation Solution for the SDS page electrophoresis

(a) **Buffer solution -Phosphate buffer**- 0.5M, 0.5M K_2HPO_4 + 0.5M KH_2PO_4 makes total volume 100ml. **Extraction buffer-** 100mM Tris-HCL pH-6.8, 4%SDS, 100mM EDTA, 10mM PMSF.**Sample solubilising buffer (SSB)-** 2.5ml of solution D + 2.5ml of 20% SDS + 2ml glycerol + 0.1ml EDTA (0.1M in water)

(b) Preparation of chemicals

12%, 4.2ml sterilized water + 3.3 ml. solution A + 2.5 ml of solution B

Solution A (30%Acrylamide w/v + 0.8%Bisacrylamid w/v) (29.2gm acrylamide, 0.8gm bisacrylamide dissolved in 100ml distilled water)

Solution B- 2.5ml (18.17gm tris (1.5%, pH-8.8) + 2ml of 20% SDS)

(75ml. tris-HCl 1.5%, pH-8.8 + 2ml. SDS (20%) + 23ml. distilled water)

APS -After mixing20-40µl (40µl) of 10%APS (Ammonium per Sulphate)

(APS- 100mg APS dissolve into 1ml distilled water)

TEMED- (N,N,N,N-tetra methyl ethylien di amine)- 10µl

APS and TEMED should be added in last and just after gel plate selling. Concentration of APS and TEMED might be change according to environment temperature.

Preparation of staking gel:-

6.1ml distilled water +1.3 ml. solution C + 2.5 ml. Solution D

Solution C- (30%w/v Acrylamide + 1.6% Bisacrylamide) (29.2 acrylamide + 1.6 gm of bisacrylamide dissolved in 100ml water)

Solution D- (6.06gm, 0.5% tris (pH-6.8) +2ml of 20% SDS)

APS -After mixing20-40µl (40µl) of 10%APS (Ammonium per Sulphate) (APS- 100mg APS dissolve into 1ml D.W.)

TEMED- (N,N,N,N-tetra methyl ethylien di amine) -10µl

APS and TEMED should be added in last and just after solidify the running gel. Concentration of APS and TEMED might be change according to environment temperature.

Reservoir (Electrode) buffer: (make up to 1,000 ml with distilled water). (Tris base -3.0 g + Glycine -14.4 g + SDS -1.0 g)

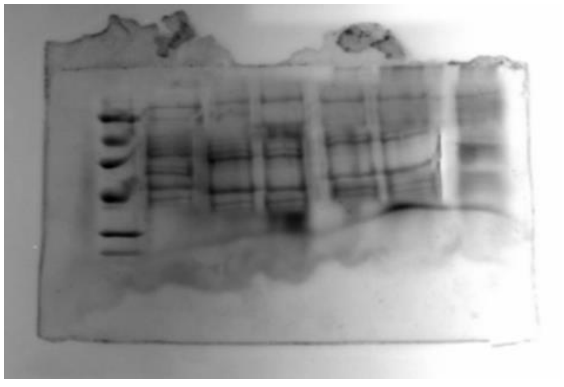
Protein Stain: Dissolved and make up to 100 ml with distilled water. (Coomassien brilliant blue-R250 - 0.25 g + Methanol -10.0 ml + Acetic acid - 7.0 ml)

Distaining solution: Mix and make up to 100 ml with distilled water. (Methanol -5 ml. + Acetic acid -7 ml. + Distilled water -88 ml.).

Sample Buffer (Sample Solubillizing buffer) 0.0625 M Tris-HCl, pH 6.8 (1.2 ml), SDS (1.0 ml), Glycerol (3.0 ml), β-mercaptoethanol(200.0µl) Bromophenol blue (1 mg ml^{-1}) (2.0 µl), Distilled water (5.0 ml)

Preparation for Bacteriocin sample

Twenty four hour old growing cultures were harvested and centrifuged at 12000rpm for 10min in cooling centrifuge at 4^0C. Supernatant was collected and Bacteriocin was precipitate with Ammonium sulphate precipitation and TC Precipitation. 100 µl of this sample was mixed with 200 µl SSB and heated at 100^0Cfor 10min. 40µl of sample was loaded in each well and run at 80V and 45 current. After completion of the gel, gel separate out from gel casting unit and staining for 1h. Then the gel was distained for over night and bserved in gel Biovis documentation unit. The molecular weight of protein bands were obtained by given method.



M.W.	Lane2	Lane3	Lane 4	Lane5	Lane6	Lane7
97400	4.444	5.00	5.714	5.714	6.666	6.714
66000	2.666	2.00	2.666	2.666	2..666	2.222
43000	1.428	1.481	2.105	1.818	1.904	1.666
29000	1.739	1.333	1.538	1.481	1.333	1.333
20100	1.379		1.333			
14300						

Result –

Table -1

Inhibition zone of bacteriocin producing strain against indicator strain in mm diameter on Nutrient agar medium.

S. No.	Bacteriocin producing strain	Indicator strain		
		E-coli 441	M78	M3
1	Bf1	13.00mm	4.4mm	4mm
2	Bf2	19.00mm	2.75mm	6.75mm
3	Bf3	18.40mm	4.0mm	4.25mm
4	Bf4	17.75mm	4.75mm	4.5mm
5	Bf5	19.66mm	6.5mm	4.0mm
6	Bf7	16.25mm	5.2mm	4.5mm
7	Bf8	18.75mm	Nil	Nil
8	Bf10	17.25mm	4.8mm	3.2mm
9	Bf11	15.00mm	2.5mm	2.3mm
10	Bf14	17.25mm	3.5mm	2.5mm
11	Bf15	14.25mm	3.5mm	Nil

12	Bf16	18.25mm	Nil	Nil
13	Jf2	15.25mm	Nil	Nil
14	Jf4	17.25mm	Nil	Nil
15	Jf6	18.00mm	Nil	Nil
16	Jf10	16.24mm	Nil	Nil
17	Jf13	18.30mm	Nil	Nil
18	Jf14	19.00mm	Nil	Nil
19	Jf15	14.00mm	Nil	Nil
20	J1	18.25mm	Nil	Nil
21	J6	17.00mm	Nil	Nil
22	J7	12.00mm	Nil	nil

< 0.5-10 mm in diameter = low susceptibility /activity

11.0-15.0 mm in diameter = moderate susceptibility/ activity

16.0-mm - > = high susceptibility /activity

The value indicate the diameter o inhibition zone in mm using 100µl of the bacteriocin in agar well diffusion on nutrient agar. The inhibition zone are mean s of triplicates.

Result

Figure 1

Figure 2

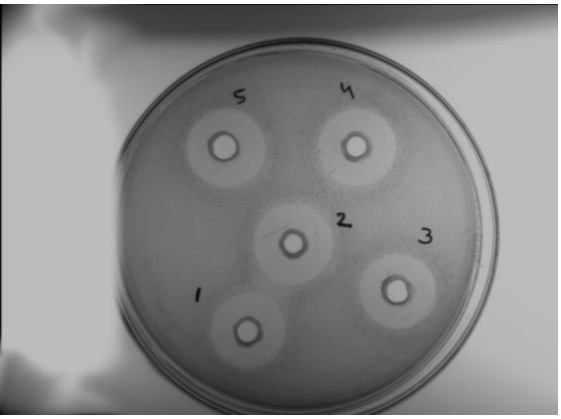
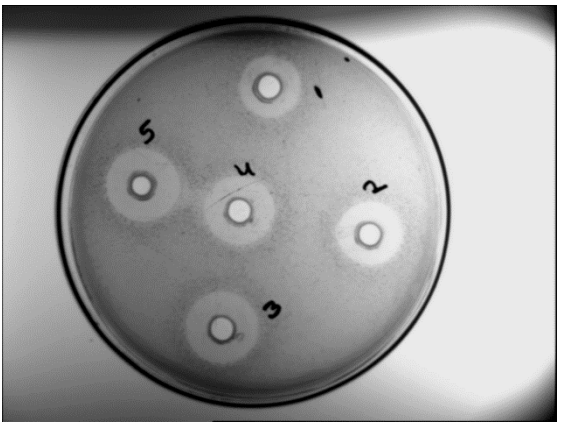


Figure 3

Figure 4



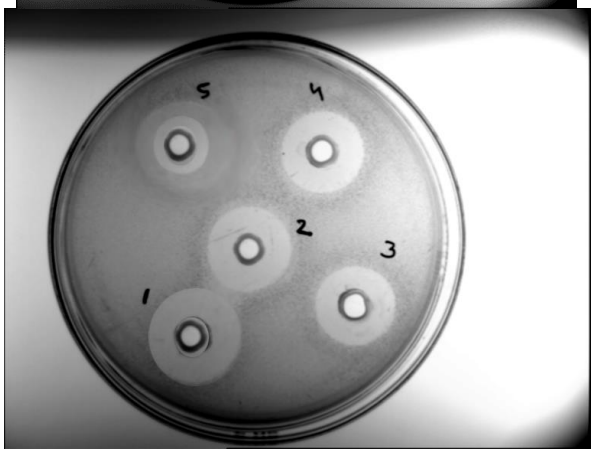
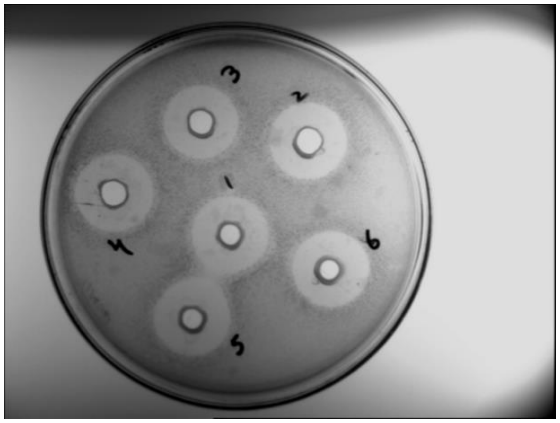


Figure 5

Figure 6



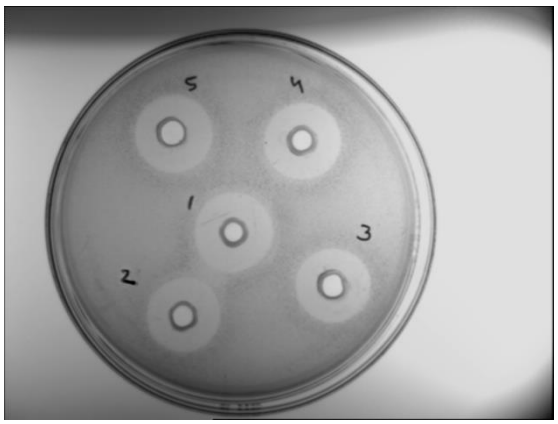


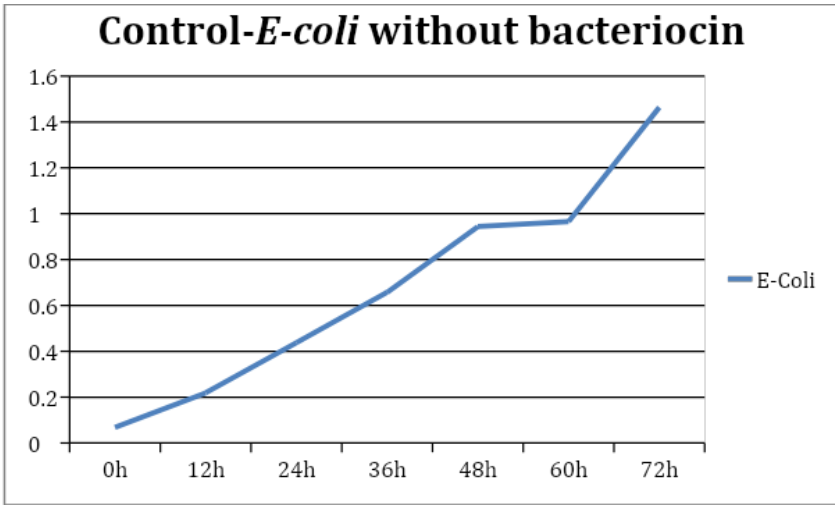
Figure 7

figure 8

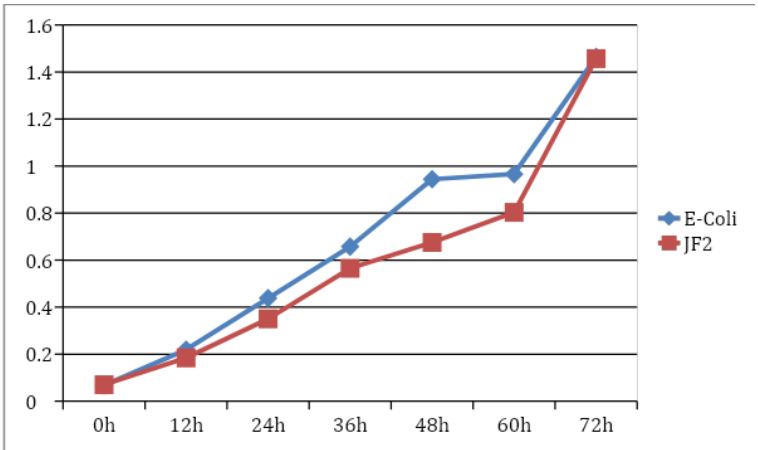
TERBITOMATRIC TEST RESUL – Growth of *E-Coli 144* with Bacteriocin producing strain.

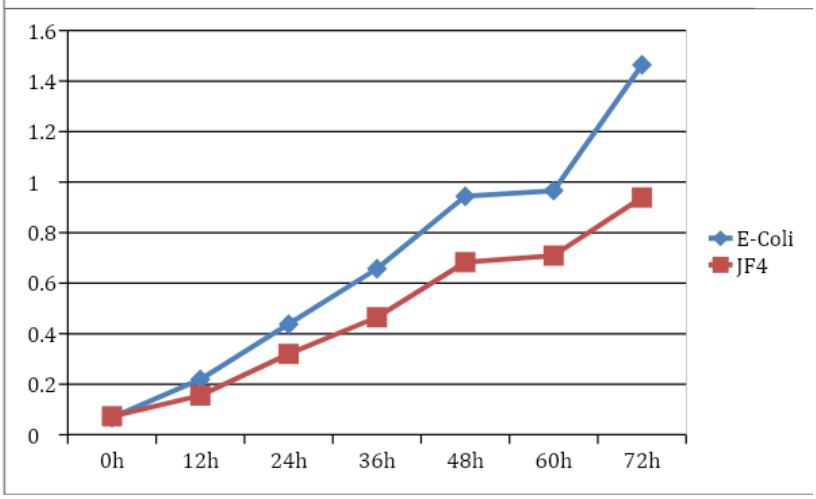
Time	oh	12h	24h	36h	48h	60h	72h
Ecoli	0.068	0.219	0.438	0.657	0.844	0.966	1.464
	0.068	0.225	0.44	0.68	0.865	0.968	1.468
	0.069	0.232	0.45	0.66	0.85	0.965	1.47
mean	0.068333	0.225333	0.442667	0.665667	0.853	0.966333	1.467333
S.D.	0.000577	0.006506	0.006429	0.012503	0.010817	0.001528	0.003055
Jf2	0.07	0.184	0.35	0.565	0.675	0.803	1.322
	0.071	0.185	0.352	0.565	0.676	0.807	1.325
	0.07	0.187	0.355	0.567	0.675	0.805	1.323
mean	0.070333	0.185333	0.352333	0.565667	0.675333	0.805	1.323333
S.D.	0.000577	0.001528	0.002517	0.001155	0.000577	0.002	0.001528
jf4	0.073	0.155	0.32	0.465	0.683	0.709	0.939
	0.072	0.155	0.321	0.465	0.684	0.709	0.939
	0.07	0.157	0.322	0.467	0.685	0.712	0.941
mean	0.071667	0.155667	0.321	0.465667	0.684	0.71	0.939667
S.D,	0.001528	0.001155	0.001	0.001155	0.001	0.001732	0.001155
Jf6	0.07	0.159	0.308	0.475	0.715	0.743	0.996
	0.07	0.158	0.309	0.474	0.718	0.742	0.997
	0.071	0.162	0.308	0.476	0.719	0.742	0.996
mean	0.070333	0.159667	0.308333	0.475	0.717333	0.742333	0.996333
S.D.	0.000577	0.002082	0.000577	0.001	0.002082	0.000577	0.000577
J7	0.072	0.161	0.337	0.483	0.773	0.759	1.111
	0.072	0.162	0.335	0.484	0.774	0.758	1.114
	0.071	0.16	0.338	0.485	0.773	0.762	1.115
mean	0.071667	0.161	0.336667	0.484	0.773333	0.759667	1.113333

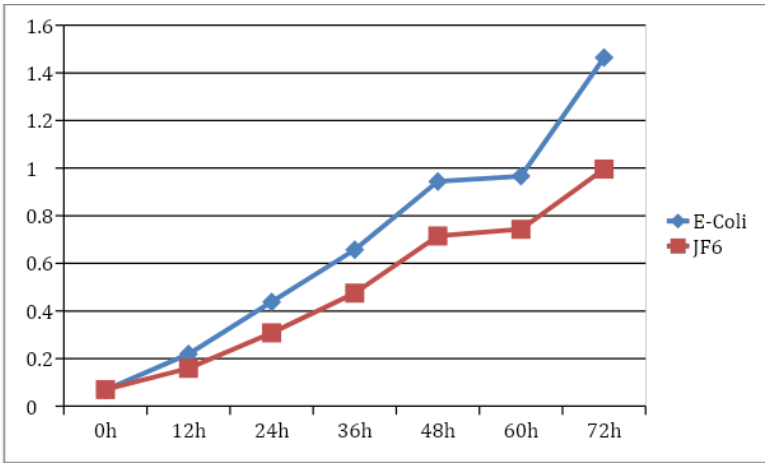
S.D.	0.000577	0.001	0.001528	0.001	0.000577	0.002082	0.002082
jf10	0.07	0.174	0.318	0.422	0.761	0.768	0.781
	0.07	0.175	0.316	0.423	0.762	0.77	0.783
	0.07	0.173	0.318	0.424	0.764	0.772	0.78
mean	0.07	0.174	0.317333	0.423	0.762333	0.77	0.781333
S.D.	0	0.001	0.001155	0.001	0.001528	0.002	0.001528
jF13	0.07	0.194	0.41	0.562	0.85	0.895	1.215
	0.071	0.196	0.415	0.565	0.853	0.897	1.218
	0.071	0.198	0.412	0.567	0.854	0.897	1.215
mean	0.070667	0.196	0.412333	0.564667	0.852333	0.896333	1.216
s.D.	0.000577	0.002	0.002517	0.002517	0.002082	0.001155	0.001732
JF14	0.072	0.189	0.288	0.54	0.842	0.852	1.199
	0.072	0.185	0.289	0.57	0.847	0.856	1.205
	0.071	0.187	0.292	0.59	0.846	0.854	1.203
mean	0.071667	0.187	0.289667	0.566667	0.845	0.854	1.202333
S.D.	0.000577	0.002	0.002082	0.025166	0.002646	0.002	0.003055
JF15	0.075	0.195	0.316	0.489	0.72	0.747	1.171
	0.074	0.198	0.31	0.488	0.725	0.748	1.173
	0.075	0.198	0.318	0.49	0.727	0.745	1.17
mean	0.074667	0.197	0.314667	0.489	0.724	0.746667	1.171333
S.D.	0.000577	0.001732	0.004163	0.001	0.003606	0.001528	0.001528
J6	0.072	0.186	0.384	0.516	0.825	0.847	1.359
	0.071	0.184	0.387	0.519	0.828	0.848	1.362
	0.072	0.187	0.383	0.521	0.83	0.852	1.365
mean	0.071667	0.185667	0.384667	0.518667	0.827667	0.849	1.362
s.D,	0.000577	0.001528	0.002082	0.002517	0.002517	0.002646	0.003



E-coli with bacteriocin JF2



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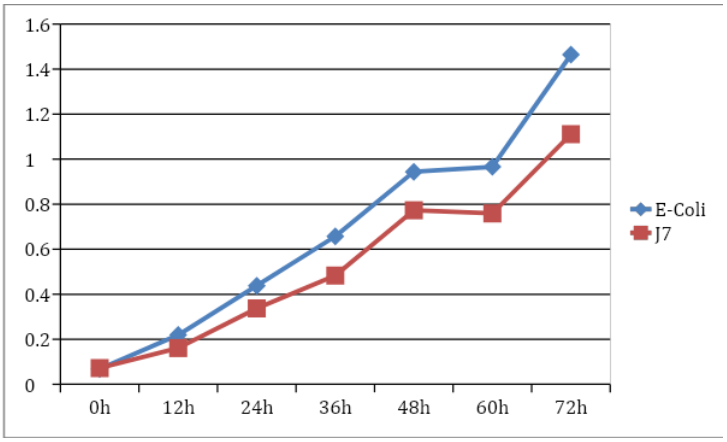
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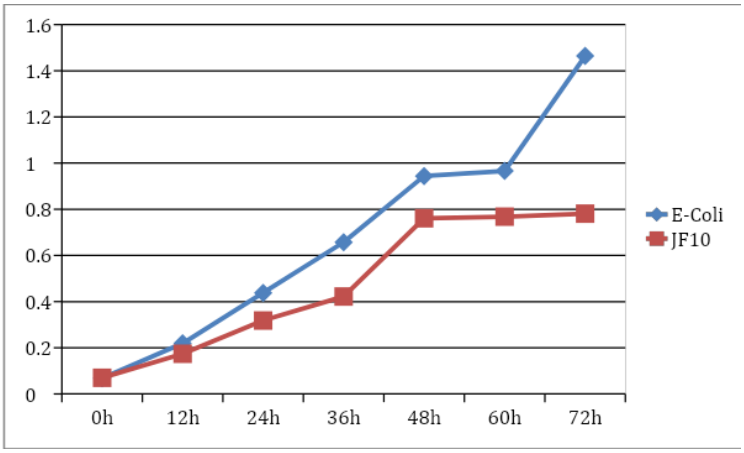
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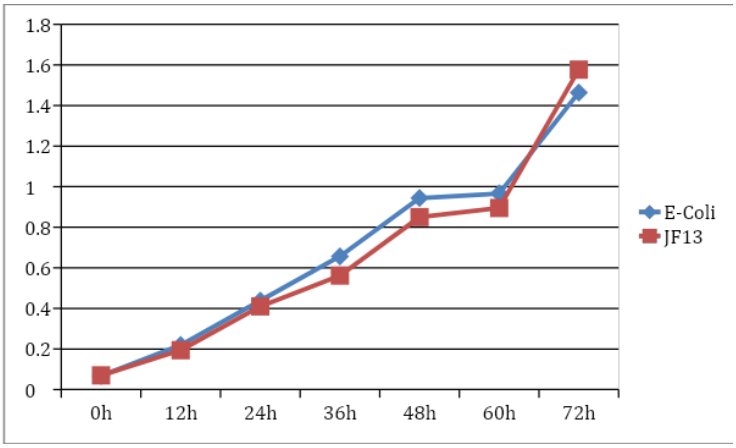
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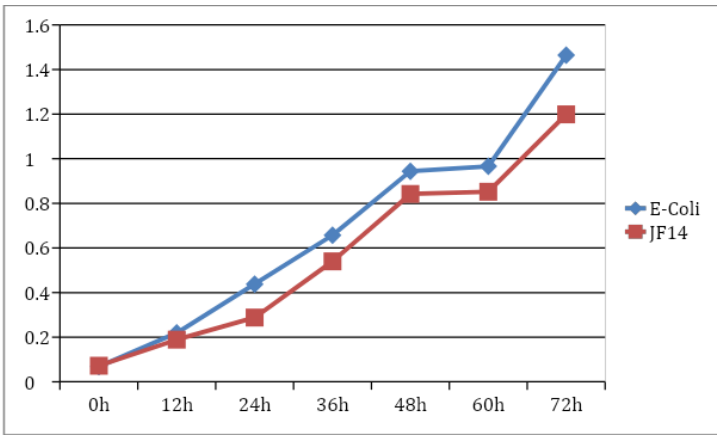
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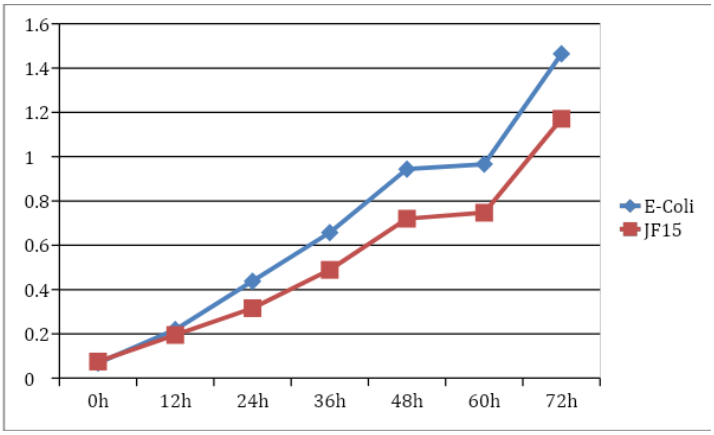
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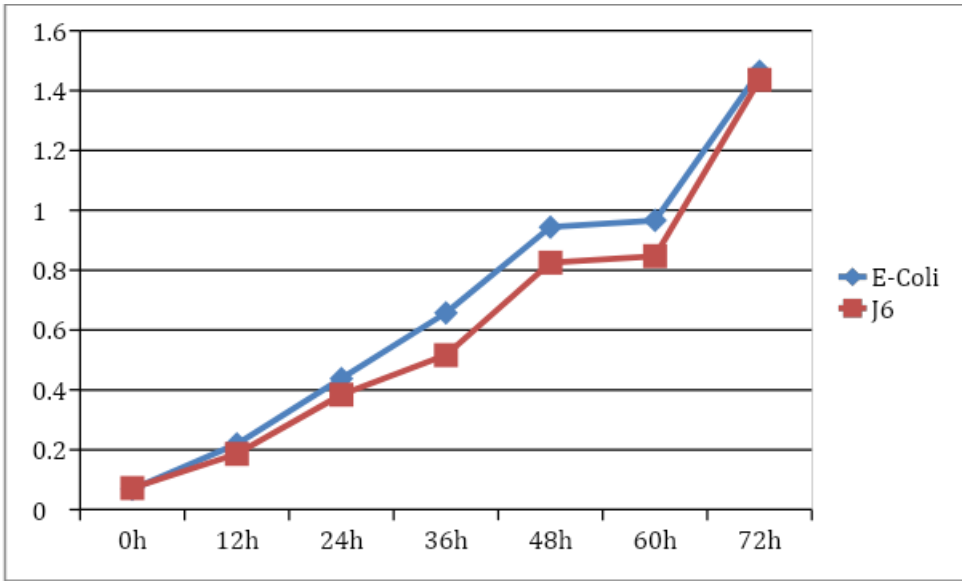

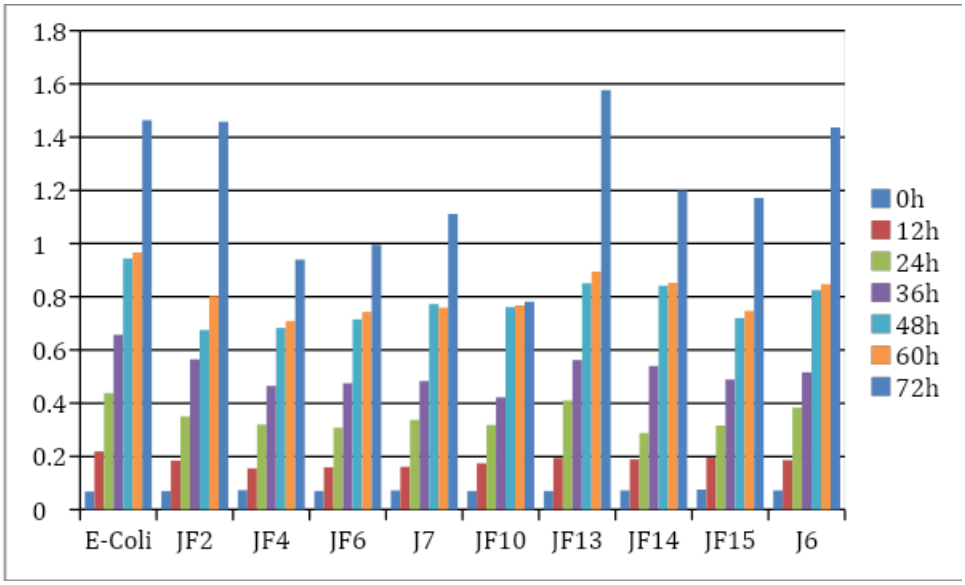

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