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BIOCONVERSION OF PHENOLIC ACIDS BY PROBIOTIC LACTIC ACID BACTERIA

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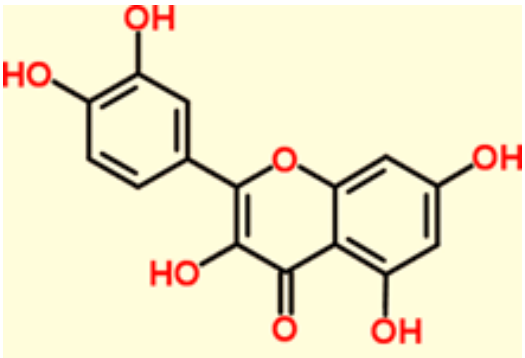
Increased levels of accumulation of toxic waste products in environment, demands for bioconversion. The advantages of bioconversion process have been well established over chemical technologies, moreover, no hazardous by-products are generated. Literature advocates tremendous scope of bioconversion in pharmaceutical industries. Phenolic compounds are important substances with widespread occurrence in plants and therefore in the human diet, known for several their beneficial influence on human metabolism. However, bioconversion of these compounds results in the formation of high value-added and costly industrial metabolites with enhanced bioactivity. Many benign microbes that are capable of degrading phenolic acids have been isolated in the last few decades, with the objective of screening an established probiotic (preferably Lactobacillus strain) which is used in the food industry. Bioconversion of phenolic acids gives valuable products like phenolic glycosides. This work is an attempt to screen and select LAB for their phenolic acid degrading capabilities and carryout bioconversion studies with the the most effective strain(s) followed by characterization. The idea was to primarily determine the bioconversion efficiency of isolated bacterial probiotic species, followed by identification of products formed during bioconversion using analytical tools TLC and HPLC. Finally, shake flask optimization was carried out for maximizing bioconvertion of phenolic acids.

Keywords: Bioconversion, Phenolic acids, Lactobacillus, Characterization, Thin layer chromatography

INTRODUCTION

Flavonoid glycosides are widespread in the plant kingdom and thus common in human diet. Pro-healthy properties of flavonoids, including their sugar deriva-tives, make them promising ingredients of dietary sup-plements and biomedical preparations. Abundantly occurring examples of flavonoids, include apigenin, eriodictyol, 3-hydroxyflavone, kaempherol, luteolin, naringenin, taxifolin quercetin and rutin. Quercetin (Figure 1) is a plant-derived antioxidant flavonoid widely distributed in red wine, green tea, onions, apples and leaf vegetables. It has displayed a variety of biological activities, including anticancer (Paliwal *et al.*, 2005), anti-inflammatory (Rotelli *et al.*, 2009), antiviral (Kaul *et al.*, 1985), and antihypertensive (Larson *et al.*, 2012)

Figure 1: Structure of Phenolic Acid Quercetin



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properties. It has been reported that it decreases oxidative DNA damage induced by reactive oxygen species, such as H_2O_2 and tert-butylhydroperoxide (Reiter *et al.*, 2009; and Alía *et al.*, 2009).

Quercetin is a secondary metabolite with established health benefits. Use of chemical methods for the production of these compounds is expensive and not eco-friendly. However, bioconversions (by bacterial/plant/animal cells) have been known to possess several biological activities (Shimoda *et al.*, 2007; and Shimoda *et al.*, 2008). Microbial transformation is a powerful approach to modify the structures of bio-active natural flavanoids to its derivatives (Shah S. Chapter 1. 1-42; Hapter C. Chapter 1). Employment of microbial transformations to obtain new derivatives of this flavonoid has many advantages of highly selective operation at optimum pH, and temperature with minimum or reduced levels of toxic waste products (Perkins, 2015).

Varied conditions for effective bioconversion have been cited in the literature. Under conditions whereby flavonoids are toxic, e.g., when they are present in high concentrations, certain microorganisms could produce various enzymes (e.g., glycosyltransferase) transforming the phenolic compounds into less toxic metabolites, as it was shown e.g., for glucansucrase of *Leuconostoc mesenteroides* acting on luteolin, quercetin and myricetin (Bertrand *et al.*, 2006), cellulase of *Penicillium decumbrens* on quercetin (Chen *et al.*, 2011). Various reactions like glycosylation, deglycosylation, methylation, glucuronidation, ring cleavage, etc. are involved in the bioconversion process of quercetin. Such processes have potential indus-trial application, due to their relatively low costs and mild reaction conditions. Noteworthy is that products obtained by microbial transformation of flavonoids are classified as natural compounds, which facilitates their potential application as food supplements or ingredients of cosmetics and pharmaceuticals. Because flavonoid are consumed in appreciable amounts in our diet, knowledge of their pharmacological and physiological properties is of significant importance. Therefore, the present study has objective to test the possibility of getting new transformed products that might have a medical importance to human.

Literature states that quercetin can be converted into quercetin-40-O-methyl-7-O-b-D-glucopyranoside and quercetin3-O-b-D-glucopyrranoside by the filamentous fungi *Beauveria bassiana* ATCC 7159 and *Cunninghamella elegans* ATCC 9245, respectively (Miyakoshi *et al.*, 2010; and Araújoa *et al.*, 2013). Bac-teria *Bacillus cereus* transformed quercetin to isoquer-cetin (quercetin 3-*O*-glucopyranoside) with 20.0% yield (Rao *et al.*, 1981). Also Quercetin, improved several key fermentation traits (like accelerated fermentation of various sugars and lactic acid production) for the performance of *L. plantarum* in food production (Landete *et al.*, 2014).

Lactic Acid Bacteria [LAB] are a physiologically diverse group of organisms, which can be generally described as Gram-positive, nonsporing cocci or rods with lactic acid as the major product of carbohydrate fermentation. *Lactobacillus* species are selected for the bioconversion process because they are non-pathogenic, benign, safe to handle and also the bioconverted products will not have any harmful effect (Macfarlane and Cummings, 1999; and Juodeikiene *et al.*, 2012). Extensive studies have revealed that many species of LAB (majorly belonging to *lactobacillus*) have promise for improving human health and preventing disease (Muhammad *et al.*, 2018; and Zieliñska *et al.*, 2018). Since *Lactobacillus* contains abundant natural product biosynthetic enzymes, some of its endogenous enzymes may be able to accept quercetin as a substrate to generate modified compounds, despite this, they were never tested for the conversion of some phenolic acids. The current work aimed at studying the catalytic activity of several *Lactobacillus* strains upon enzymatic reactions, to obtain different metabolites. Herein, the bioconversion of quercetin by *Lactobacillus* species has been reported.

MATERIAL AND METHODS

Bacterial Strain and Growth Conditions

The lactic acid bacterial strains for carrying out the present study were procured from National Facility for Biopharmaceuticals (NFB), G.N.Khalsa College.

The four strains procured were *Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. Bulgaricus, Lactobacillus fermentum and Lactobacillus delbrueckii subsp. Lactis* showing Percent identity 99%, 91%, 91%, 99% with reference sequences having NCBI accession no. MG654777, MH100982, MH100902, MH205691 respectively. Stock cultures were maintained in Lactobacillus Selection Media (LSM) vials at 4 °C and sub cultured for inoculum preparation, using LSM and incubating at 37 °C at 180 rpm

Primary Screening for Bioconversion of Phenolic Compounds

Primary screening was done to evaluate the selected cultures (established lactic acid bacteria) for their bioconversion

capabilities. The screening was designed in such a way that cultures with no or lower activity were discarded, thus identifying the isolates with required bioconversion capabilities. A 'plate assay' based on agar cup diffusion method was performed. Twenty five mL Sterile Minimal media agar (composition Table 2) was incorporated with sterile 0.2%, 0.4% and 0.6% quercetin, Wells of 5 mm diameter were bored into the plate with a sterile cork borer and about 100 µL culture suspensions were added to the wells. Overnight grown cultures of *L. acidophilus, L. bulgaricus, L. fermentum* and *L. lactis* ($OD_{660\,nm}$ adjusted to 0.5) in sterile minimal media was used for the purpose. All the plates were incubated at 37 °C for 48 hours. Zones of the exhibition (mm) were measured after incubation. The concentration showing maximum zone were identified to be optimal for bioconversions and selected for further study.

Verification of Strains

The ability of selected strains to transform quercetin was assessed using the following procedure: Test strains were inoculated into their corresponding broth with 0.2%, 0.4% and 0.6% quercetin and incubated at 37° for 48 h. Broths were centrifuged at 5000 rpm for 3 min at RT. To the supernatant equal volume of 2% $AlCl_3$ (w/v) dissolved in methanol was added and left to react for 10min, after which the absorbance of solution was recorded at 415 nm in a UV-VIS spectrophotometer . Sterile broth containing quercetin was used as the negative control. The strain with highest bioconversion ability was selected for the further bioconversion studies.

Spectrophotometric quercetin degradation assay was used to determine the final concentration of quercetin for given cultures.

Quercetin Degradation Assay

Stock solution of quercetin of concentration 1000 µg/mL was prepared by using ethanol as a diluent and then further diluted to 100, 200, 400, 600 and 800 µg/mL. The reaction mixture was prepared according to the Table 1, mixed well. Blank was prepared in similar way by replacing aluminium chloride with distilled water and their absorbance was measured against the blank at 510 nm. Colour change of standard quercetin were seen yellow to red orange. Absorbance of test sample were recorded in similar way.

Study for Bioconversion

Sterile Minimal medium broth (containing quercetin) was used as the bioconversion medium for the study. The media was optimized for bioconversion study of 0.2% of quercetin particularly as Quercetin tends to precipitate easily at higher concentrations. Sterile stock solutions of all the components were prepared in water and diluted during the studies as per the requirement. A common media base was prepared as 10% sterile stock solutions of quercetin (in Dimethyl sulfoxide, i.e., DMSO). For the study, the stock solutions were added to the media base to give a final concentration of 0.2% for quercetin.

The above-prepared media were inoculated with 0.2 mL overnight grown culture ($OD_{660\,nm}$ = 0.5) and incubated at 37 °C for 96 h at 180 rpm.

Extraction of the Bioconverted Products

Extraction of the bioconverted products was carried out after 96 h incubation of by centrifuging the broth at 7000 rpm for 10 minutes at RT. Supernatant so obtained was subjected to 3 rounds of liquid-liquid extractions.

Table 1: Protocol for Quercetin Degradation Assay

Conc. of Stock (µg/mL)	Vol of Stock (µL)	Vol of Diluent (µL)	Total Vol (mL)	Distilled Water (mL)	5% Sodium Nitrate (µL)	Incubate for 5 mins at RT	10% Aluminium Chloride (µL)	Sodium Hydroxide (1%) (mL)	O.D. was Recorded at 510 nm
100	100	900	1	4	300		300	2	
200	200	800							
400	400	600							
600	600	400							
800	800	200							
1000	1000	-							
Blank	-	-							

Table 2: Media Composition		
Component	**Quantity for Stock (g/10 mL)**	**Volume to be Added for 100 mL Media (mL)**
Na_2HPO_4	1.41	2.33
K_2HPO_4	1.72	1.62
NH_4Cl	0.53	0.94
$MgSO_4.7H_2O$	2.46	0.1
Glucose	2	2.5
Trace Elements Solution		0.1
$FeSO_4.7H_2O$ (2.78g/L), $MnCl_2.4H_2O$ (1.12g/L), $CaCl_2.2H_2O$ (1.67g/L), $ZnSO_4.7H_2O$ (0.29g/L).		
Dissolve in 0.1M HCl.		
pH		6
Sterile distilled water		277.23

Equal volume of ethyl acetate was used as optimized solvent to extract the quercetin from 300 mL of supernatant. The extracted solvents were evaporated completely at 60 p C and then resuspended in 2 mL methanol for quercetin.

Analysis and Characterization of the Bioconverted Product

For the analysis and characterization of the bioconverted products obtained by extractions technique was used – High Performance Thin Layer Chromatography (HPTLC)

HPTLC—It was used as a primary and quick characterization method. In this, TLC plates pre-coated with silica (Silica Gel F254) were used. The resuspended sample was loaded along with the standards quercetin and controls. Five µL of the samples and 2 µL of the standard were loaded on the plate as spots and run in a TLC with the mobile phase.

Table 3: HPTLC Parameters			
Phenolic Compound	**Standard Used**	**Mobile Phase**	
		Composition	**Ratio**
Quercetin	0.1% quercetin in methanol	Toluene : Ethyl acetate : Formic acid	5.4.0.2 (v/v/v)

The plates were run 80% of 10 cm and then analyzed under UV at 254 nm. The Rf values for all the bands were calculated.

RESULTS AND DISCUSSION

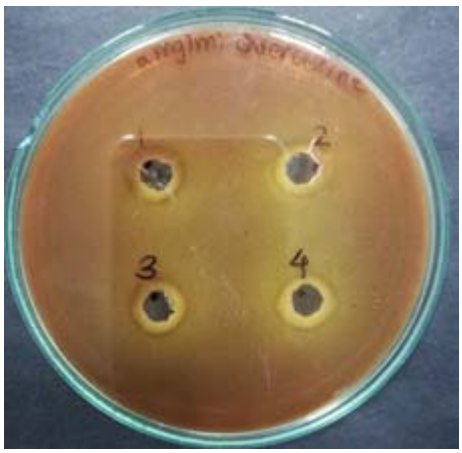
Primary Screening for Bioconversion of Phenolic Compounds

Zones of exhibition were measured to screen the cultures and identify the bioconversion capabilities of the strains (Table 3). Larger zone size indicated that the isolate could tolerate 0.2% quercetin quite well as compared to other isolates and on the same basis concentration of quercetin was selected. *L. bulgaricus* showed the largest zone of 23 mm diameter followed by *L. lactis* of 20 mm, *L. fermentum* of 18 mm and lastly *L. acidophilus* of 17 mm.

Primary screening of the isolates for their potency of bioconversion revealed that all the 4 isolates used had the ability to tolerate the 0.2% concentration of the quercetin

Table 4: Zone of Clearance (in mm) Exhibited by Selected Cultures				
S No.	**Culture**	**Quercetin Concentration (in %)**		
		0.2	**0.4**	**0.6**
1	*L. acidophilus*	17	-	-
2	*L. bulgaricus*	23	16	16
3	*L. fermentum*	18	-	-
4	*L. lactis*	20	9	-

Figure 2: Plate Assay for Quercetin Degradation



and thus bring about the bioconversion. On the basis of primary screening, 0.2% concentration was selected for the bioconversion of thus, primary screening proved to be beneficial in the selection of optimal concentration of quercetin that can be bioconverted.

Verification of Strains

Bioconversion studies were carried out for 48 h with 0.2% concentration of quercetin, with the 4 cultures: *L. acidophilus, L. bulgaricus, L. fermentum* and *L. lactis.*

In the case of quercetin, the media colour change was an indication of bioconversion. The colour change was due to the difference in the λ_{max} of the substrate quercetin and the bioconverted product(s) formed. The samples were withdrawn at time intervals of 24 h, 48 h and 72 h. Although most of the research articles suggest that an incubation of 72 h to be done for the bioconversions, in the current work, some strains exhibited bioconversions as early as 48 h. After 48 h media colour of quercetin changed from yellow to brown. No further colour change was observed even after extending the incubation for more than 72 h. The chemical origin of the compound(s) responsible for the appearance of intensive colour of media and isolated dry products remains unknown. It is reasonable to assume that these compound(s) should include molecular fragments chromophores.

Using spectrophotometric assays the concentration of quercetin was plotted against the corresponding absorbance resulting in a standard graph which was used to determine the concentration of quercetin in unknown samples (Figure 7). The % degradation for quercetin at 48h was calculated for individual cultures.

Figure 3: Media at 0 h

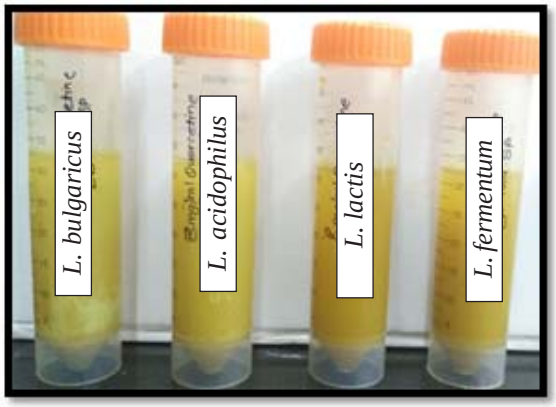


Figure 4: Media at 24 h

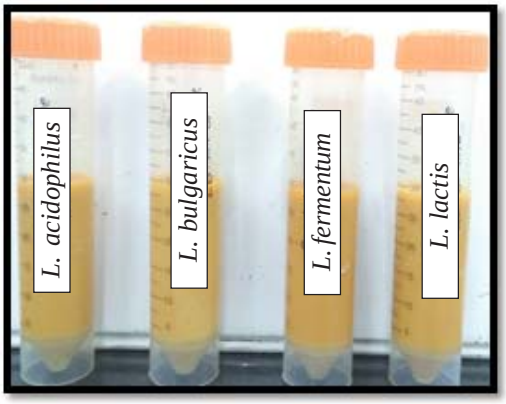


Figure 5: Media at 48 h

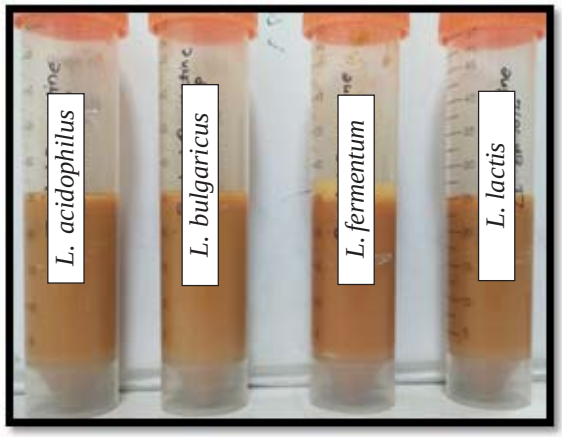


Figure 6: Media at 72 h

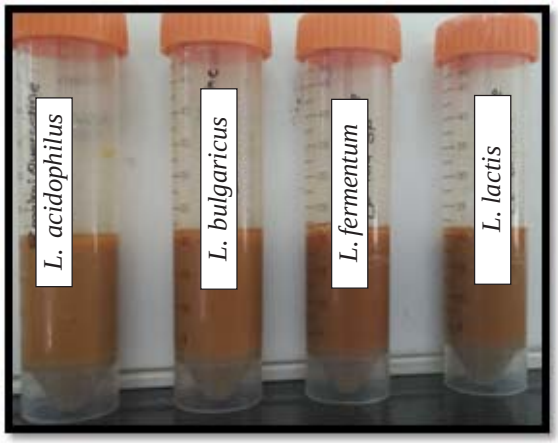


Figure 7: Standard Graph of Quercetin

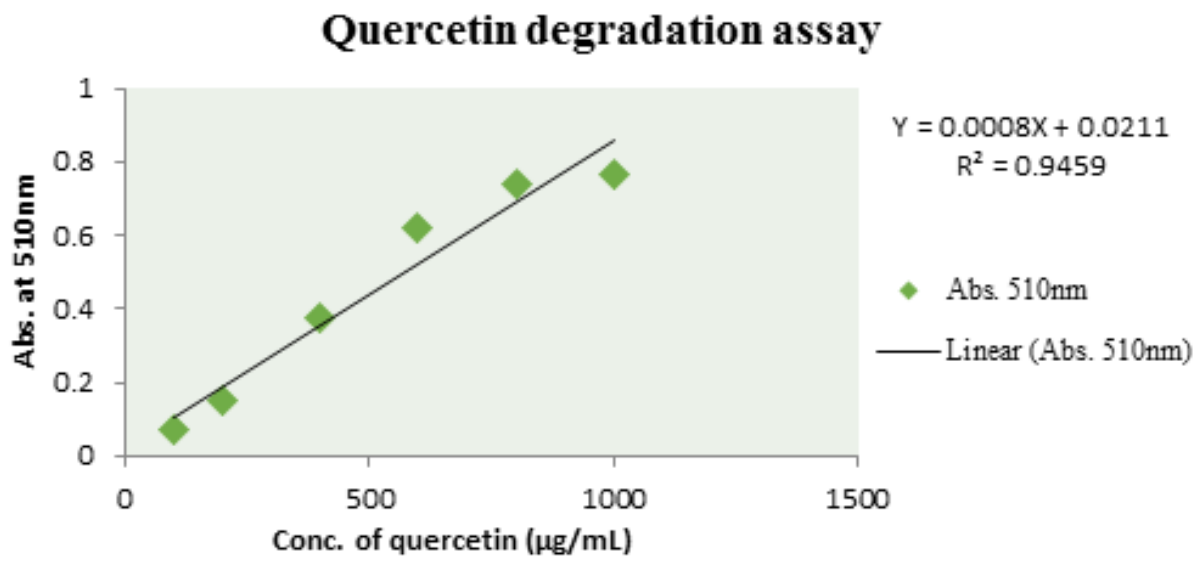
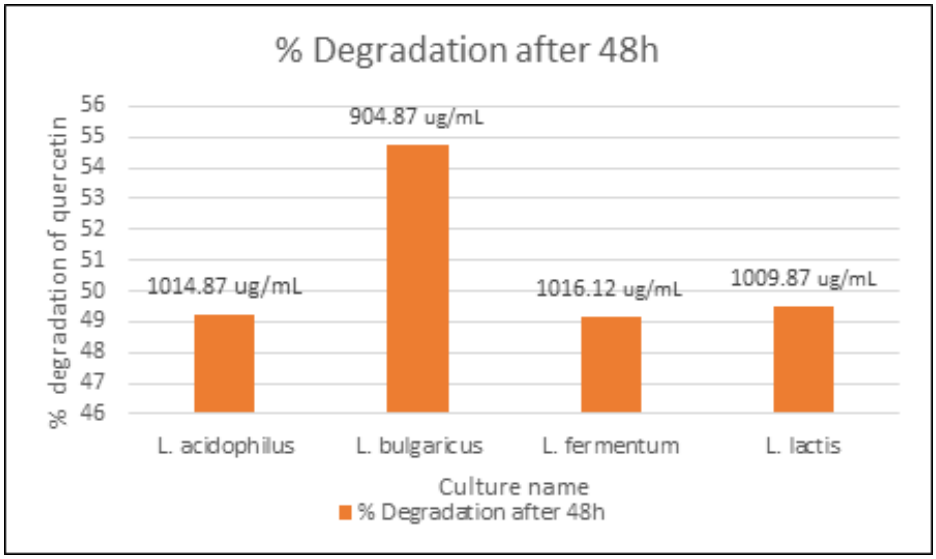


Figure 8: Quercetin Degradation Assay for Samples



The Beer's law was found to be obeyed in a concentration range (100-1000 µg/mL). The standard graph between known concentration of quercetin and absorbance is shown in Figure 7. The linear regression equation was found to be Y = 0.0008X + 0.0211, with correlation coefficient (R^2 = 0.9459) in which Y meant the absorbance value while X represented the concentration of the quercetin solution in ìg/ml. An equations (Equation 1) was formed using values of linear regression equation, at selected 510 nm wavelength. The final concentrations of quercetin in media containing individual culture after 48 h were calculated using Equation 1.

Formula

$$Conc.(\mu g/mL) = \frac{Abs - 0.0211}{0.0008}$$

$$\%\ degradation = \frac{Initial\ conc. - Final\ conc.}{Fixed\ initial\ conc.}$$

Initial conc. = 2000 µg/mL (0.2%)

$$Final\ conc. = \frac{Abs.(510\ nm) - 0.0211}{0.0008} \quad ...(1)$$

Culture *L. bulgaricus* showed highest % degradation of Quercetin (54.75 %), i.e., was able to efficiently bio convert at 0.2% concentration of quercetin and therefore, was considered potential for further studies. While other *lactobacillus* strains, i.e., *L. acidophilus, L. fermentum* and *L. lactis* showed 49.25%, 49.19%, 49.5% ability to bioconvert quercetin respectively, which are approximately similar, possibly indicating that the enzyme(s) involved for their respective bioconversion may have similar catalytic ability.

Study for Bioconversions

Bioconversion studies were carried out with 300 mL media containing 0.2% quercetin 48 h, with culture *L. bulgaricus* due to it's highest bioconversion ability compared to other strains. For the study of bioconversion, the optimized minimal media base used efficiently promoted the growth as well as allowed the bioconversion of the incorporated phenolic compound. After two days, media colour for quercetin changed from yellow to brown.

Figure 9: Media at 0 h

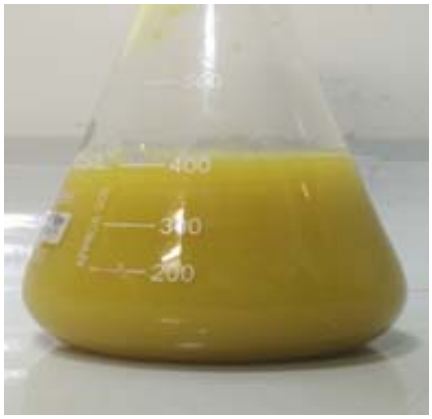


Figure 10: Media at 48 h

Analysis and Characterization of the Bioconverted Product

After incubation, the bioconverted products were extracted using liquid-liquid extractions with suitable solvents. The products were more soluble in the organic solvents used than the media. Multiple extractions of the media supernatant increased the efficiency of extractions.

The dry extracted solid product from the broth was dissolved in 2 mL methanol and 5 µL of this sample was loaded on TLC plate using CAMAG Linomat 5, HPTLC instrument.

Thin-layer chromatography was performed on 5 cm x 10 cm aluminium plate pre-coated with Silica Gel F254 (Merk). Bands of (each 8 mm in length) of 2 µL standard quercetin of concentration 0.1% and 5 µL methanol extracts of fermentation media were applied in Lane 1 and Lane 2 respectively, 1 cm above the edge of the plates by use of CAMAG Linomat 5, HPTLC instrument. Plate was developed to a distance of 9 cm from the origin by ascending chromatography in a rectangular CAMAG glass chamber containing 10 mL mobile phase Toluene: Ethyl acetate : Formic acid (5:4:0.2 v/v/v) and previously saturated for 10 min at RT. After 10 min, plate was removed, dried in air and visualised under UV light at 254 nm by use of CAMAG TLC scanner 3. The bands with R_f value 0.39, 0.28, 0.15 are referred as band A, B, C respectively for simplicity.

In the sample, a total 4 bands were obtained, with R_f values as 0.35, 0.39, 0.28, 0.15. In both standard and sample a band of R_f 0.35 was obtained which showed maximum absorption at 372 nm and hence were confirmed to be of quercetin. All the remaining 3 bands obtained in sample had a different R_f value than the standard quercetin band. This showed that bioconversion of quercetin occurred using given culture. The compound present in the band A with R_f value 0.39 showed maximum absorption at 262 nm, while in band B with R_f value 0.28 showed maximum absorption at 261 nm and in band C with R_f value 0.15 showed maximum absorption at 382 nm.

The number of multiple bands signifies the presence of bioconverted product in the media as a qualitative analysis.

Figure 11: HPTLC

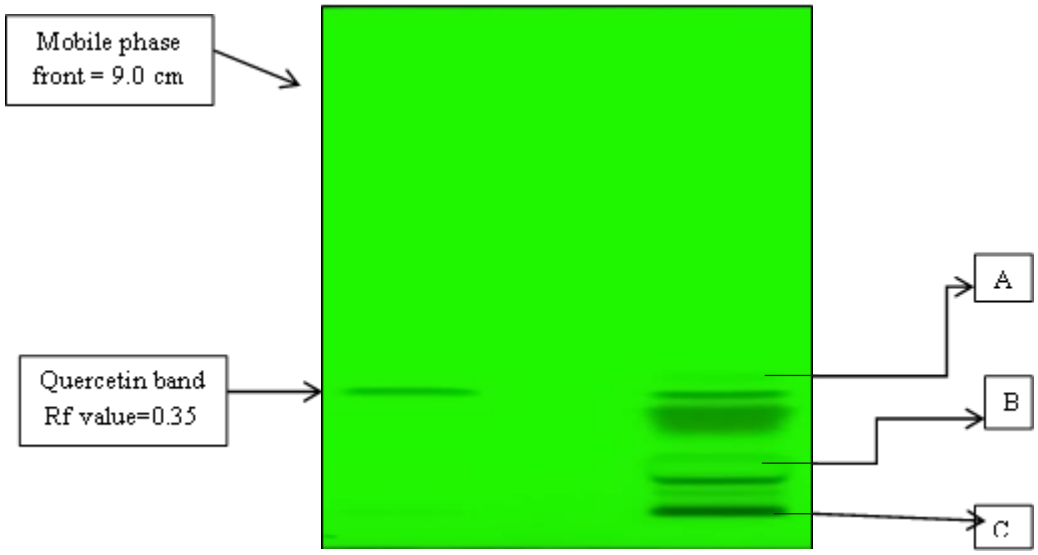
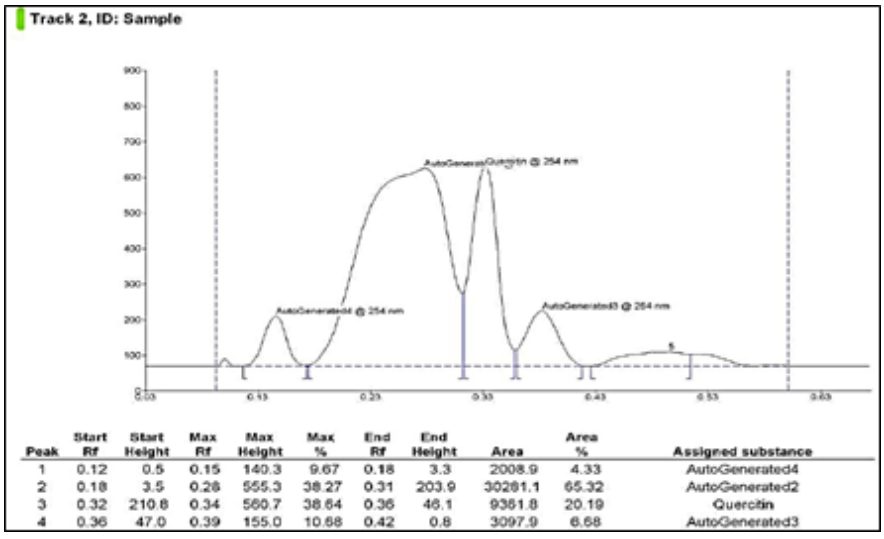


Figure 12: Quantitative Analysis Result of HPTLC Showing Amount of Converted Products Present in Our Media with Culture



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.12	0.5	0.15	140.3	9.67	0.18	3.3	2008.9	4.33	AutoGenerated4
2	0.18	3.5	0.26	555.3	38.27	0.31	203.9	30281.1	65.32	AutoGenerated2
3	0.32	210.8	0.34	560.7	38.64	0.36	46.1	9361.8	20.19	Quercitin
4	0.36	47.0	0.39	155.0	10.68	0.42	0.8	3097.9	6.68	AutoGenerated3

For more specific and quantitative analysis its HPTLC was done.

HPTLC used for the characterization of bioconverted products and was a quick method and gave reliable results. In the case of quercetin, *L. bulgaricus* gave four bands (Figure 5), a band with R_f value equal to that of the quercetin standard and 3 other band indicating that quercetin was bioconverted most probably into one of its derivatives.

CONCLUSION

Primary screening of the isolates demonstrated the capacity of *L. acidophilus, Lactobacillus bulgaricus, L. fermentum, L. lactis* for the bioconversions of the phenolic, viz., quercetin. The bioconverted products accumulated in the medium after 48 hours. Therefore, an incubation of 48 hours was proved to be appropriate for the bioconversions to occur. Extraction of the bioconverted metabolites was done

efficiently using liquid-liquid extractions with suitable solvents. Product characterization was done successfully by HPTLC. HPTLC showed that the *Lactobacillus bulgaricus* convert quercetin into three different quercetin derivatives.

With all the results, it can be concluded that the screening and evaluation of the potent *Lactobacillus* for the bioconversion of quercetin was done successfully. Bioconversion of the phenolic compound quercetin, was done successfully using the bacterium *Lactobacillus bulgaricus.*

Studies to explore the quercetin derivatives formed by bioconversion using Fourier-Trans form Infrared Spectroscopy (FTIR) and Mass Spectrometry (MS) and their identification as well as analysis of metabolic pathways involved in bioconversion to form derivatives can be done. Strain improvement can be applied to study the bioconversion and check the capability of the wild types and mutant strains for the bioconversion of other phenolic compounds. Conducting studies that will enhance the bioconversions by employing a mixed culture (consortium) of *Lactobacillus* and comparing the bioconverted products formed with that formed by the pure cultures can be achieved. Strategies can be made to check the efficacy of the *Lactobacillus* strains (wild-type and mutants) to be used as probiotic food.

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