UTILIZATION OF PINE NEEDLES AS BED MATERIAL IN SOLID STATE FERMENTATION FOR PRODUCTION OF LACTIC ACID BY *LACTOBACILLUS* STRAINS

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Pine needles, which are abundantly found as underexploited biomass in coniferous forests, are responsible for fire hazards and air pollution. Utilization of pine needles as bed material in lactic acid production with solid state fermentation (SSF) has been studied here. This investigation compared lactic acid production by pure strains of Lactobacilli, (1) *L. delbrueckii* (NCIM2025); (2) *L. pentosus* (NCIM 2912); (3) *Lactobacillus sp.* (NCIM 2734); (4) *Lactobacillus sp.* (NCIM2084); and a co-culture of the first two strains. The studies required 6 g per flask powdered dry pine needles as bed material, 2 g/L (inoculum), liquid production media based on pure glucose or whey substituted glucose, at 60, 80, and 120 g/L sugar levels, 37 ^{o}C, and an initial pH of 6.5. Co-culture attained a maximum lactic acid concentration of 45.10 g/L, followed by that of strain-1, 43.87 g/L and strain-4, 26.15 g/L, in 80 g/L pure glucose media. With 120g/L total sugar in whey-substituted media, the co-culture attained maximum lactic acid production of 44.88 g/L followed by that of strain-1, 43.67 g/L. The present experimental studies indicated better compatibility of pine needle bed with co-culture in solid state fermentation of lactic acid, which may prove to be an eco-friendly technology for utilization of biomass as well as minimizing fires in coniferous forests.

Keywords: Co-culture; Lactic acid; Lactobacilli; Pine needles; Solid state fermentation

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INTRODUCTION

The enormous amounts of fallen pine needles, usually found as waste biomass in coniferous forests, have been often associated with devastating forest fires. These fires are one of the dominant ecological events that occur periodically and pose formidable threat for the animal and plant life in the coniferous forests. Pine needles bear immense potential in the future through their utilization as bed material in solid state fermentative production of value-added chemicals, such as lactic acid. Lactic acid, a hydroxy carboxylic acid, is produced mainly through fermentation. It is used as feedstock for production of the eco-friendly biodegradable polymer polylactic acid (PLA), in food or dairy industries as an acidulant, preservative, or flavoring agent, and also as probiotics and bacteriocins (through its microbes) (Adsul et al. 2007; Narayanan et al. 2004; Faris et al. 2007). The Lactobacilli also exhibit several antagonistic and biocontrol properties suitable for agricultural, food, and health protection purposes (Ghosh and Ghosh 2009a).

The pines are coniferous, evergreen, resinous trees belonging to the genus *Pinus* of the family *Pinaceae*, native to the northern hemisphere. They are also found in south-east Asia and the Himalayan regions of India. The Chir pine (*Pinus roxburghii*) is predominant in coniferous forests of the Himalayan regions of Uttarakhand and Himachal (Kala 2004). The needle-shaped green colored adult pine leaves found in clusters are known as pine needles. They constitute the major portion of the litter fall in coniferous forests (Merila and Derome 2008). Pine needles have several applications, in addition to their role in returning nutrients to the forest floor. They can serve as a mulching material, a supplier of nutrients when added to soil, and as a beverage (with high amounts of vitamins A and C), pine needle wine. Pine needle powder is a rich source of essential oils, vitamin C, and alkaloids. Pine needles can help in biomonitoring of pollution by absorption of atmospheric polycyclic aromatic hydrocarbons (PAHs) (Kim and Chung 2000; Judzentiene et al. 2006; Ratola et al. 2006). Kwak et al. (2006) experimentally established that the ethanol extracts of pine needles (*Pinus densiflora*) exhibit, strong antioxidant, antimutagenic, antiproliferative properties and antitumor effects under in vitro and in vivo conditions. Pine needles have also been applied as reinforcing agents in place of conventional ones in polymer composites, offering several advantages such as lower cost, lesser density, non-corrosiveness, reduced wear in process equipment, improvement in mechanical properties, biodegradability, and recyclability, etc. (Thakur and Singha 2010a,b). Pine needles of length 170 to 250 mm, diameter 0.7 to 1.3mm, or in dried ground particle form (200 μm), containing ca. 33.4% lignin, 67.3% holocellulose, 2.71% ash, and 15% extractives, have been used in preparation of polymer-based biocomposites with resorcinol formaldehyde matrix, urea formaldehyde resin, and phenol formaldehyde, resulting in improvement of mechanical properties such as tensile strength, compressive strength, etc. (Singha and Thakur 2010b, 2009a, 2010c, 2009b, 2010d).

A high risk of forest fires, associated with dried, fallen, pine needles, results in large-scale destruction of fauna and flora and is hazardous to the environment due to air pollution (Singha et al. 2008). Cannac et al. (2007) reported that roughly $50x10^3$ fires per year destroy about $6x10^5$ hectares of forest per year in Mediterranean islands (France); these fires mainly involve Corsican pine (*Pinus nigra ssp laricio*) found abundantly in mountains of Corsica. Consumption of pine needles by cattle, especially during late stages of pregnancy, can cause premature parturition, in which the calf dies after birth (Panter et al. 1992).

Entry of tannins from pine needles in the soil was found to inhibit the growth of various beneficial agricultural microbes, causing delayed availability of nutrients in soil (Selvakumar et al. 2007). Selvakumar et al. (2007) discussed that the water-soluble polyphenolic compounds (tannins) in pine needles affect the soil nutrient dynamics by delaying the organic matter decomposition and mineralization of nutrients. The experimenttal study further showed that nitrogen fixing microbes (*Rhizobium* and *Azotobacter*), phosphate solubilizing bacteria *Bacillus halodurans* and cellulose decomposing fungi *Trichoderma reesi* and *T.virescens* were inhibited at 50, 1000, 100, and 3000 ppm of water extracted tannins, respectively. Hence this finding clearly indicated that tannins from the pine needles hinder the organic matter decomposition and nutrient availability to the forest soil. The used up pine needle bed materials after fermentation and subsequent lactic acid extraction step, contains some of the residual sugars from the production

media and the bed materials (where a portion of the pentosan fraction degrades during autoclaving), traces of salts (sodium, potassium, and phosphate, etc.) and has reduced quantities of phenolic substances (lignin and tannins) due to washing and extraction. Such used up, ground pine needle bed material may be deposited back to the forest soil, where due to larger surface area of ground pine needle particles, reduced tannins and other phenolics, and additional sugars, microbial decomposition can progress more readily to release nutrients, eventually helping in restoration of soil fertility in forests. This can serve as a long term measure to avoid nutrient depletion from forest soil along with lactic acid production utilizing pine needles as bed materials. Enormous amounts of pine needles can be considered as under-exploited biomass in the coniferous forests of Himachal Pradesh and Uttarakhand states of India. Hardly any research work has been performed regarding the utilization of pine needles, the abundant biomass in coniferous forests, as bed material in solid state bacterial production of lactic acid.

Various cheap and abundant carbohydrate-rich waste materials have been applied for solid state bacterial lactic acid production, such as cheese whey and potato starch hydrolyzate (Ghosh et al. 2010). Cheese whey, a dairy byproduct, has a BOD of 38,000 to 46,000 ppm and COD 80 g/L due to the presence of approximately 5% lactose, 0.06% fat, and 0.8 to 1% nitrogenous compounds (Bullerman and Berry 1966). It has global production of about 10^8 tons/year and is very expensive to treat by the conventional activated sludge process; however its lactose component can be bioconverted into valuable fermentation products such as organic acids, ethanol, and single-cell proteins (Ghasemi et al. 2009; Panesar et al. 2007).

The objectives of the present experiments were to investigate (1) the feasibility of solid-state bacterial fermentation in lactic acid production utilizing a bed of powdered pine needles for growth and production through various *Lactobacillus* pure strains and co-culture, (2) to observe the effectiveness of dairy whey, as a cheap substitute for expensive pure sugars, (3) to determine the effect of various doses of pure glucose in pure and dairy whey substituted glucose media on production of lactic acid, and (4) to evaluate the advantages of using the co-culture over its constituent strains.

EXPERIMENTAL

Material and Methods

Chemicals used in the work were of Merck and High Media make. Pure cultures of Lactobacilli strains (1) *L. delbrueckii* (NCIM2025), (2) *L. pentosus* (NCIM 2912), (3) *Lactobacillus sp.*(NCIM 2734), and (4) *Lactobacillus sp.* (NCIM2084) were acquired from National Chemical Laboratory (NCL), Pune, India. Pine needles were acquired from the Dhanolti area near Mussoorie district of Uttarakhand state, India, while the cheese whey was procured from a nearby dairy. The composition for one liter of MRS (de Mann Rogosa Sharpe) culture media used for inoculum preparation of the Lactobacilli strains was: 10 g proteose peptone, 5 g yeast extract, 10 g beef extract, 20 g dextrose, 1 g Tween 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4$, and 2 g K_2HPO_4 in distilled water as solvent. Each liter of glucose-based synthetic production media contained: various levels of glucose (60, 80 and 120 g/L) in pure glucose-based

production media and 30, 40, and 70 g/L in whey-substituted media; 1 g sodium acetate; 0.03 g $MnSO_4.H_2O$; 0.10 g $MgSO_4.7H_2O$; 15 g yeast extract; 0.25 g KH_2PO_4; 0.25 g K_2HPO_4, and 0.03 g $FeSO_4$. Components, other than total sugar, were kept the same in both cases. NaOH, at the 2% level, was used as neutralizer instead of 2% $CaCO_3$, the neutralizer usually used, due to the inhibitory effects of calcium carbonate previously investigated on the bacterial strains under study (Ghosh and Ghosh 2008, 2009b).

Pentosan assay in pine needles

Pentosans in the wood and pulp can be determined by TAPPI method T223 cm-01 based on the principle of conversion of pentosans to furfural (in the distillate) by boiling with HCl. The amount of furfural is quantified colorimetrically. Based on a similar principle a modified spectrophotometric method for determination of the pentosan content in the non-wood (agricultural/forest residues) or woody materials has been mentioned in the TM1-A11 test method of the laboratory manual of Central Pulp and Paper Research Institute (CPPRI), Saharanpur, U.P., India. Absorbance of the distillate in the receiver resulting from acid digestion of 3 g OD (oven dry) sample in 300 mL 13.5% HCl (at boiling condition) contained in a round-bottomed flask connected with pentosan apparatus, was measured at 280 nm. During digestion the acid level in the round-bottomed flask was maintained by addition of HCl drops through a separating funnel at the top of the flask. Pentosan percentage was evaluated by the following expression (Laboratory manual 2001):

Pentosan (%) = Absorbance at 280 nm x Dilution x 1.563 x 0.5 x

100/151 x OD weight of the sample (1)

Estimation of lignin content in pine needles

Lignin was quantified as per the TM1-A-7 test method mentioned in the laboratory manual of CPPRI (Laboratory manual 2001) similar to the T222 om-06 TAPPI standard method for Klason lignin (insoluble lignin) estimation. It consists of addition of 2 mL 72% sulphuric acid to 1 g OD sample, followed by addition of 13 mL of the same acid in the same beaker that was placed in a water bath at 20 ^oC with continuous stirring for 2 hours. The contents of the beaker were filtered through a G2 crucible that weighed (W_1). The crucible along with the residues was dried overnight in an oven at 105 ^oC and weighed (W_2). The acid-insoluble lignin was evaluated by the following expression:

Acid insoluble lignin, % = (W_2-W_1) x 100/OD weight of the sample (2)

The filtrate from the G_2 crucible was kept for soluble lignin estimation, which was measured spectrophotometrically at 280 nm. Acid-soluble lignin can be evaluated through the following expression (Laboratory manual 2001):

Acid soluble lignin, % =Absorbance at 280 nm x Dilution x

100/20/1000 x OD weight of the sample (3)

Estimation of holocellulose content in pine needles

This method quantifies the total carbohydrate content of woody or non-woody materials, as per the TM1-A-9 test method mentioned in the laboratory manual of CPPRI (Laboratory manual 2001) that is analogous to the holocellulose determination method mentioned by Goncalves et al. (2005). 5 g of ground OD material (passed through mesh size 40) was placed in a 250 mL Erlenmeyer flask with 10 mL distilled water, followed by the addition of 1.5 g of sodium chlorite and 0.5 mL of acetic acid. The mixture was incubated in a flask for one hour at 70 ^{o}C in a water bath. The supernatant was transferred to a tared crucible (W_1). The contents of the conical flask were filtered in a tared crucible, followed by washing of the residue with acetone and oven drying at 105 ^{o}C for two hours. The crucible with dried contents weighed (W_2).

Holocellulose % can be calculated by the following expression (Laboratory manual 2001):

$$\text{Holocellulose, \%} = (W_2 - W_1) \times 100 / \text{OD weight of the sample} \quad (4)$$

Determination of ash content

Five grams of oven-dry pine needles sample (ground to pass through mesh size 40) were taken in a sintered crucible and then placed inside a muffle furnace at 600 ^{o}C for two hours, followed by cooling in a dessicator. Ash content was determined by the following expression:

$$\text{Ash, \%} = W_2 \text{ (weight of ash with crucible)} - W_1 \text{ (crucible weight)} \times 100/\text{OD weight of sample} \quad (5)$$

FT-IR analysis

Fourier transform infrared spectroscopy (FT-IR) was performed using a Nicolet-6000 spectrophotometer to ascertain the presence of some of the functional groups associated with compounds in the bed material. Dried ground pine needle samples were mixed with potassium bromide and pressed under vacuum. Transmittance values were measured over a range near 500 to 4000 cm^{-1}.

Inoculum preparation

The inoculum preparation for the *Lactobacillus* strains under study was performed in standard MRS media. For each strain, a loop full of pure culture acquired from National Chemical Laboratory, Pune (India) was transferred from MRS agar stabs to the 50 mL of MRS liquid media contained in Erlenmeyer flasks under aseptic conditions. In case of the co-culture control sample, a loop full of each from strain-1 and strain-2 were transferred to the MRS media. All the inoculated flasks were screw-capped tightly and then kept in an incubator shaker at 37 ^{o}C and 180 rpm for 14 hours.

Bacterial cell concentration

Equal volumes of culture broth were filtered through preweighed microporous filter (0.7μm) papers that were washed with 0.85% sterile saline solution prepared in

distilled water and dried at 85 °C until attainment of constant weight. The bacterial biomass was measured in gram per liter (g/L).

Lactose estimation

Lactose content of the cheese whey was estimated by a colorimetric method (Nickerson et al. 1976). The absorbance of the sample was evaluated at 540 nm.

Preparation of production media

The preparation of the pure glucose-based media involved the use of pure glucose 60 g, 80 g, and 120 g per liter media as carbon source. In whey-substituted glucose media 30 g, 40 g, and 70 g of pure glucose was used per liter media, the rest of the sugar being supplied from whey containing 5% lactose to make total sugar concentrations of 60 g, 80 g, and 120 g per liter. The rest of the media components remained the same, with an initial pH of 6.5. 2% NaOH was used as neutralizer.

Preparation of bed material

Powdered pine needles, weighing 6 g were uniformly dropped in each of the clean oven-dried 250 mL conical flasks after prior washing and drying. The particle size distribution of the bed material is shown in Table 3.

Solid state fermentation process

40 mL of the production medium and 1.0 mL of 2% NaOH neutralizer were added uniformly to each of the conical flasks containing 6 g bed material and autoclaved for sterilization. After cooling, these were inoculated with different strains of Lactobacilli, keeping the input biomass concentration at 2 g/L. The flasks were screw-capped (with sterile cotton packing in the lower and upper sides of the capping) immediately under aseptic conditions and kept for incubation at 37 °C in an incubator for a period of six days.

Extraction and assay of lactic acid

After six days of incubation, 50 mL of distilled water was added to each conical flask and shaken for two hours at 200 rpm. The resulting mixture was passed through a muslin cloth, into which the solid bed particles were removed. The pH values of the extracts were recorded by a digital pH meter and subsequently assayed for lactic acid. The quantitative assay of lactic acid present in the extracts was performed by the Kimberly Taylor method. The absorbance for lactic acid was measured with a UV-VIS double beam Systronics 118 spectrophotometer at 570 nm (Taylor 1996; Mirdamadi et al. 2002).

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed using a LEO-435VP microscope to obtain clearly magnified views of the growth of *Lactobacillus* cells on the bed material. Very small portions of bed material dried at 60 °C, for 30 hours, were placed on the stubs and subjected to gold coating and observed at 15 kV.

RESULTS AND DISCUSSION

Studies on the Ground Pine Needles Bed Material

The major components such as holocellulose, pentosan and lignin in ground pine needles bed material can affect the growth and the resultant production of lactic acid, obtained from the pure cultures and coculture of *Lactobacilli* under study. The values of these components are given in Table 1.

Table1. Composition of Major Constituents of Ground Oven-Dried Pine Needles

Analyzed constituents of pine needles	Weight %
Holocellulose	64.12 ± 2.68
Pentosan	14.12 ± 1.76
Lignin	27.79 ± 1.8
Ash	3.24 ± 0.09

Results expressed as mean ± standard deviation, based on the repeated trials

The particle size of the bed material exerts a significant impact on growth and production of microorganisms, in solid state fermentation. The surface area, liquid holding capacity, and the gaseous exchange depend upon the size of bed material particles. In this case, the size of the ground pine needles had particle sizes lesser than 150 μm and up to 1204 μm. A major portion (by weight) of powdered pine needle particles used here was greater than 420 μm, followed by those of greater than 500 μm and lesser than 150 μm. If the bed is composed predominantly of very small sized particles, it has large surface area but reduced mass transfer (Rojan et al. 2006b).

Table 2. Particle Size Fractions of Dried, Ground Pine Needle Bed Material Obtained by Sieving through Vertically Arranged Screens in Order of Diminishing Pore Sizes in a Vibratory Shaker Operated for 10 Minutes

Mesh size (microns)	Weight% of the particles retained
1204	3.63
750	11.57
710	9.82
500	17.85
420	26.61
210	7.02
150	7.43
Fine residual particles	16.07

In the present study, very small sized particles such as of 210 and 150 μm size constituted the minimum portions by weight; hence larger particles of size greater than 420 microns being predominant provided better mass transfer for the production media, inoculum, and neutralizer, etc. The finer particles lesser than 150 microns, which constituted the third highest fraction as indicated in Table 2, enhanced the surface area for good growth of bacteria on the surface of bed, which was clearly observed in Fig. 4: 4(b), 4(c), 4(d), 4(e), and 4(f).

Lactic Acid Production on Pine Needles Bed Material

In Table 3, the *Lactobacillus* strains 1, 4, and co-culture attained the maximum individual lactic acid concentrations of 43.87 g/L, 26.15 g/L, and 45.10 g/L, respectively, at 80 g/L dose of pure glucose as carbon source, while strains 2 and 3 reached 13.075 g/L and 13.87 g/L at 120 g/L and 60 g/L pure glucose levels, respectively, in the production media. Thus, co-culture achieved the highest lactic acid production among all the strains with 80 g/L pure glucose application. The lowest dose of glucose, 60 g/L proved to be the most suitable for strain-3, where it attained its maximum individual concentration of lactic acid. Inhibition due to high sugar level (120 g/L pure glucose) was evidenced in Table 3, where a sharp decline in lactic acid production occurred in all the bacterial strains, except, strain-2. Although the strain-2 could grow and utilize glucose (hexose) but being primarily a pentose sugar consumer, it was not significantly inhibited by higher dose of glucose (hexose). It may, additionally, consume some of the pentose sugars available, due to hemicellulose degradation from pine needle bed, during autoclaving; hence it showed its highest lactic acid concentration of 13.075 g/L at 120 g/L pure glucose.

Table 3. Lactic Acid Production by Different Strains of Lactobacilli Corresponding to Various Doses of Pure Glucose in the Production Media.

Bacterial strains	Glucose (60 g/L)		Glucose (80 g/L)		Glucose (120 g/L)	
	pH	Lactic Acid (g/L)*	pH	Lactic Acid (g/L)*	pH	Lactic Acid (g/L)*
L.delbrueckii (NCIM2025) Strain-1	4.22 ±0.051	35.97±0.78	4.08 ±0.048	43.87±1.08	4.42 ±0.054	32.56±0.72
L.pentosus (NCIM2912) Strain-2	6.10 ±0.036	3.37±0.13	5.89 ±0.022	8.72±0.23	5.34 ±0.035	13.08±0.34
Coculture of first two Strains	4.20 ±0.046	36.71±0.79	3.90 ±0.025	45.10±1.18	4.48 ±0.058	34.84±0.76
Lactobacillus sp. (NCIM2734)Strain-3	5.32 ±0.030	13.87±0.37	5.88 ±0.036	9.53±0.25	6.02 ±0.028	5.74±0.16
Lactobacillus sp. (NCIM2084)Strain-4	5.92 ±0.023	7.00±0.18	4.46 ±0.057	26.15±0.63	5.98 ±0.025	4.56±0.14

* in extract (Results given as mean ± standard deviation, based on repeated trials)

The higher production of lactic acid at 120 g/L glucose treatment by the strain-1 *Lactobacillus sp.* (NCIM2025) and co-culture as per Table 3 indicated their better capability to withstand the high level sugar inhibition better than other strains and carry out a reasonable level of lactic acid production. Among all the bacterial strains at the lowest glucose level (60 g/L) in Table 3, the co-culture showed the highest lactic acid formation 36.71 g/L (pH 4.20), closely followed by strain-1 with 35.97 g/L (pH 4.22) lactic acid. In Table 3, the co-culture showed better acid production (lower pH) than its constituent strains (1) and (2) at 60 and 80 g/L doses of glucose; thus its application may prove better at the lower and the optimum doses of glucose. In Table 4, all the bacterial strains and co-culture attained their individual maximum values for lactic acid production at 120 g/L total sugar level, in whey-substituted glucose media, where the co-culture displayed the highest lactic acid production of 44.88 g/L. The increase in lactic acid production with increase in total sugar level, given in Table 4, indicated no significant sugar inhibition at 120 g/L total sugar level. This observation contradicted the trend of

inhibition observed in Table 3, at 120 g/L pure glucose. This may be due to the presence of nitrogenous compounds and vitamins in whey, which stimulate more cell growth, leading to better utilization of sugars. In Table 4, the co-culture exhibited the highest lactic acid production at 120 g/L total sugar level, which was higher than that of the constituent strains1 and 2. The co-culture was more beneficial due to the dual advantage of utilizing disaccharides (lactose) and monosaccharides hexose (glucose), as well as pentose sugars (possibly liberated during autoclaving, from the mean value 14.12% of pentosan fraction of pine needle bed material, as mentioned in Table 1).

Table4. Lactic Acid Production by Different Strains of Lactobacilli Corresponding to Various Doses of Glucose Substituted with Whey Lactose, in the Production Media.

Bacterial strains	Glucose 30 g/L+ whey lactose 30 g/L		Glucose 40 g/L+ whey lactose 40 g/L		Glucose 70 g/L + whey lactose 50 g/L	
	60 g/L		80 g/L		120 g/L	
	(pH)	Lactic Acid (g/L) *	(pH)	Lactic Acid (g/L) *	(pH)	Lactic Acid (g/L)*
L.delbrueckii (NCIM2025)Strain-1	4.18 ±0.038	37.21± 0.80	4.14 ±0.033	39.46± 0.82	3.98 ±0.031	43.67± 1.10
L.pentosus (NCIM 2912) Strain-2	5.98 ±0.026	5.19± 0.13	5.86 ±0.021	8.02± 0.24	4.81 ±0.056	22.08± 0.54
Coculture of strain1,2	4.41 ±0.050	27.77± 0.69	4.12 ±0.030	40.84± 0.91	3.93 ±0.028	44.86± 1.10
Lactobacillus sp. (NCIM2734)Strain-3	6.20 ±0.047	1.28± 0.10	5.90 ±0.032	7.89± 0.18	5.04 ±0.050	18.02± 0.48
Lactobacillus sp. (NCIM2084)Strain-4	5.92 ±0.024	5.73± 0.15	5.18 ±0.036	10.02± 0.27	4.45 ±0.058	26.45± 0.67

*in extract (Results given as mean ± standard deviation, based on the repeated trials)

In Table 4 co-culture showed the highest pH drop (3.93), closely followed by strain-1, pH 3.98. Each strain including the co-culture attained its lowest pH values with 120 g/L total sugar level, which is in accordance with their highest lactic acid production values in Table 4. The slightly acidic pH accompanied with high temperature (100 to 120 ^{o}C) and pressure conditions during autoclaving might have possibly degraded the acetylated xylans from the bed material into xylan and acetic acid (Kumar et al. 2010a). The breakdown of hemicellulose may have been due to the autohydrolysis or dilute acid hydrolysis reactions, possible during autoclaving. Hydronium ions required for both the processes may either be present initially or could be generated later through the autoionization of water. These subsequently depolymerize hemicelluloses, by hydrolysis of glycosidic linkages and acetyl groups. In the second stage of hydrolysis, hydronium ions from the generated acetic acid have greater impact on hydrolysis than those available from autoionization. These catalyze the hydrolysis of links between hemicelluloses and lignin. The autohydrolysis may convert hemicelluloses into soluble monosaccharides or oligosaccharides, with reduced amount of byproducts (Carvalheiro et al. 2008). Higher lactic acid production values by some strains also reflected their ability to withstand the low pH (acidic conditions), as in case of strain-1 and co-culture, in Table 4.

At the 120 g/L total sugar level all the strains and co-culture provided higher lactic acid production in Table 4 than Table 3 (with pure glucose). This indicated stimulatory action of vitamins and nitrogenous compounds from whey, resulting in higher growth of bacterial biomass, which enhanced sugar uptake resulting in higher lactic acid production.

Lactose Uptake by *Lactobacillus* Strains

For lactose metabolism in *E.coli*, a requirement of proteins such as β-galactosidase (for splitting lactose into glucose and galactose) and a carrier molecule lactose permease and the role of glucose inhibition in the lactose uptake and hydrolysis, through regulation of gene expression in *lac* operon, have also been reported (Freifelder 1998; Plummer 2002; Satyanarayana 2005). The lac operon has also been found in various Lactobacilli, including *L.casei* and *L.delbrueckii*, etc., for the uptake and initiation of the lactose metabolism. In some of the Lactobacilli additional uptake systems of lactose, such as the lactose-specific, phosphoenol pyruvate-dependent phosphotrans-ferase system (lac-PTS), also exist, other than the lac operon associated Lactose permease and β-galactosidase enzyme systems (Alpert and Siebers 1997). The higher production of lactic acid shown in Table 4, by the bacterial strains and co-culture at 120 g/L total sugar level, despite the possible inhibition of *lac* operon (due to higher dose of glucose 70 g/L) suggested that these Lactobacilli may probably be using other mechanisms of lactose uptake and hydrolysis under the given conditions. Some of the lower lactic acid producing strains in 120 g/L whey substituted media may be doing so, because of lesser or no utilization of galactose sugar, produced due to intracellular splitting of lactose by β-galactosidase enzyme, into equimolar quantities of glucose and galactose. A similar case has been described for *Lactobacillus delbrueckii* subsp. *bulgaricus* strains, which utilized a lactose/galactose antiport transport system to export the resultant galactose out of the cell. Only partial utilization of galactose to lactic acid was achieved (Welman and Maddox 2003).

Weight of the Lactic Acid Produced in the Pine Needle Bed Material

The results shown in Fig. 1 suggest that the co-culture gave the highest lactic acid weight of 1.836 g and 1.3882 g, closely followed by strain-1 with 1.799 g and 1.86 g per 6 g pine needle bed with pure glucose and whey substituted glucose, respectively, at 60 g/L total sugar level. The strain-1, strain-2, and co-culture enhanced their lactic acid weights with whey combination due to better compatibility with lactose sugar and growth-stimulating compounds in whey.

In Fig. 2 the same trend for highest lactic acid weight continued, as the co-culture provided the highest lactic acid weight of 2.2550 g and 2.0420 g, closely followed by strain-1 with 2.194 g and 1.973 g per 6 g bed in pure glucose and whey-substituted glucose respectively at 80 g/L total sugar level.

A decline in lactic acid weight was evidenced in Fig. 3, due to inhibition caused by high level (120 g/L) of pure glucose application in all the strains, while the same strains showed their maximum lactic acid weight at120 g/L total sugar level with whey substitution.

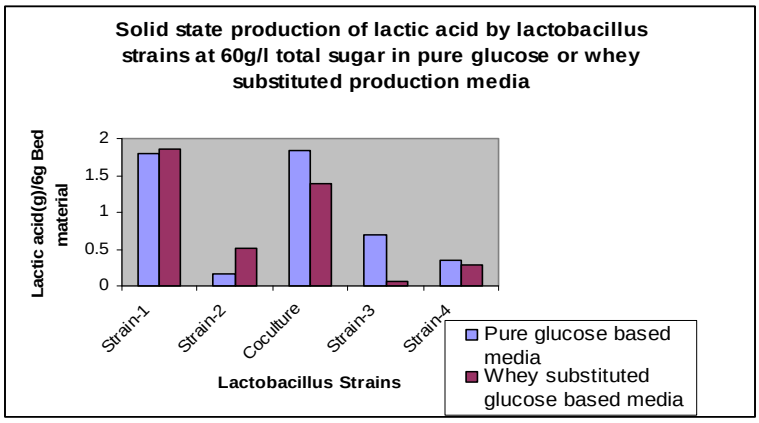

Fig. 1. Weight of lactic acid in (g/6g) bed of ground pine needles obtained with 60 g/L total sugar input after six days fermentation by *Lactobacillus* strains (mean values)

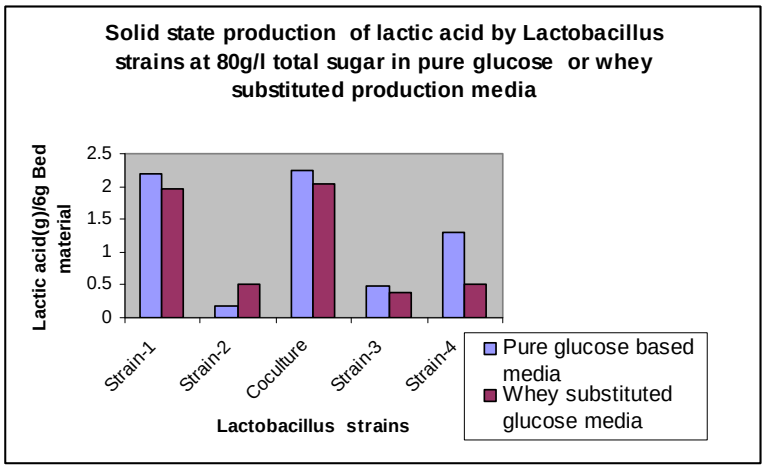

Fig. 2. Weight of lactic acid in (g/6g) bed of ground pine needles obtained with 80 g/L total sugar input after six days fermentation by *Lactobacillus* strains (mean values)

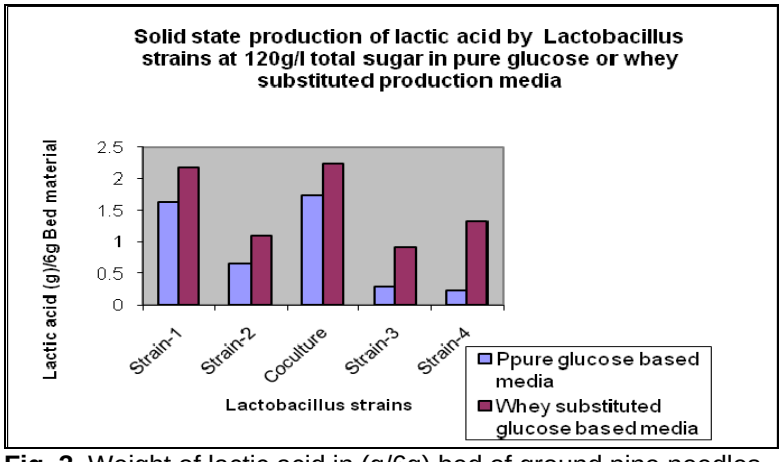

Fig. 3. Weight of lactic acid in (g/6g) bed of ground pine needles obtained with 120 g/L total sugar input after six days fermentation by *Lactobacillus* strains (mean values)

The weight of lactic acid (g)/6g bed material at 120g/L pure glucose for strain-1 through strain-4 and co-culture, were 1.6280, 0.6538, 0.2869, 0.2278, and 1.7420, respectively, while the same pure strains and co-culture provided 2.1835, 1.1040, 0.9010, 1.3236, and 2.2440 weight of lactic acid (g)/6g bed material respectively, with 120 g/L dose of sugar in whey-substituted glucose. This anomalous behavior of the bacterial strains at 120 g/L sugar dose may possibly be due to the presence of a different sugar (lactose), reduced glucose component (70 g/L), and stimulatory substances available from whey.

It was evident from the Figs. 1 through 3 that the co-culture provided the maximum weight of lactic acid per 6 g of the bed material at 80 g/L and at 120 g/L sugar level, which was followed next by the strain-1 at the same doses. Rojan et al. (2006a) reported a maximum lactic acid production of 249 mg/gds (gram dry fermented substance) through *L. delbrueckii* action on inert (washed), sugarcane bagasse bed with starch hydrolyzate in 5 days, but in the present studies, addition of production media containing whey, on pine needle bed, inoculated with *L. delbrueckii* as pure strain provided 311.92 mg/gds of lactic acid, while as co-culture it liberated 320.57 mg/gds lactic acid. Hence this observation highlighted the significance of utilizing coculture, whey substitution, and a non-inert bed.

4(a)

4(b)

4(c)

4(d)

4(e)

4(f)

Fig. 4. Growth of Lactobacilli on solid bed of pine needles. Frames 4(b), 4(c), 4(d), 4(e), and 4(f) show the growth of the strains-1, 2, 3, 4, and coculture on pine needle bed fed with a production media containing whey lactose (50 g/L), combined with glucose (70 g/L) at 120 g/L total sugar level, incubated for six days, while 4(a) shows control bed with no inoculation.

Figure 4 shows the growth patterns of different strains of Lactobacilli on the pine needle bed, added with whey substituted glucose based production media containing 120 g/L total sugar level where they attain their individual maximum lactic acid production values as observed in Table 4. Frames 4(b), 4(c), 4(d), 4(e), and 4(f) represent the growth of strains-1, 2, 3, 4, and coculture, while 4(a) shows a non-inoculated bed of powdered pine needles. Among these, the strains-1, 2, 4, and coculture displayed greyish white colored, dense growth over the bed, as observed in Fig. 4: 4(b), 4(c), 4(e), and 4(f) due to higher utilization of the vitamin and nitrogenous substances from whey, as compared to that of strain-3, seen in Fig. 4(d). The lesser growth of strain-3 observed in 4(d), consequently, resulted in its least lactic acid production of 18.02 g/L, among all the strains, as mentioned in Table 4.

Data Analysis

The data in Tables 1, 3 and 4 have been expressed as mean ± standard deviation. The Karl Pearsons coefficient of variation (CV) for the lactic acid concentration data in Tables 3 and 4, corresponding to different levels of sugar treatments, suggests that 120 g/L dose of whey-substituted glucose (with least CV), has most consistent lactic acid concentration values from different strains, with least variation. The uniformity in the production values at 120 g/L dose of whey-substituted glucose further indicated that this treatment level was most successful in enhancing the lactic acid production of all the strains under study. All of these strains attained their maximum concentration of lactic acid at this level (Table 4). The next higher value of CV was obtained with 80 g/L pure glucose application, (where only three out of five strains attained their maximum lactic acid concentration), indicated lesser consistency among the production values from different strains. A very high CV value at 120 g/L pure glucose input level indicated comparatively lesser consistency and higher disparity in lactic acid production by the bacterial strains under study, as a consequence of inhibition in lactic acid production (Table 3) due to highest dose of sugar application. Very high CV thus suggests that the corresponding input level of pure glucose (120 g/L) ceased to be beneficial for production of lactic acid. The correlation analysis between the weights of lactic acid in bed and input of lactose from whey (Table 4) provided positive correlation coefficient (γ), which suggests interdependence and positive non-linear correlation between these variables. The t-test analysis carried out on the lactic acid production data by different bacterial strains (Table 4), justified the fact that the rising sugar inputs along with whey lactose did not have any significant sugar inhibition affecting lactic acid production. The above statistical finding is further complemented by the fact that whey is known to contain stimulatory constituents for the Lactobacilli, which are helpful in their growth and production.

Growth of *Lactobacillus* Strains in Inhibitory Environment

The presence of a wide range of inhibitory alcohols and phenols, such as ethyl alcohol, isobutyl alcohol, iso-amyl alcohol, phenyl ethyl alcohol, benzyl alcohol, phenyl propyl alcohol, and 2-methoxy 4-vinyl phenol, have been reported in pine needle tea (Kim and Chung 2000). The compound 3-vanillyl propanol has been detected as the predominant phenolic compound in *Pinus laricio*, followed by phenyl ethyl alcohol, and

dihydro p-coumaryl alcohol, etc. (Cannac et al. 2007). Selvakumar et al. (2007) described tannins (water soluble polyphenolic compounds) to be most important among the phenolic compounds in the pine needles (*Pinus roxburghii*).

Kumar et al. (2010a) have reported that lignin was depolymerized and repolymerized as well, on application of high temperature and acidity to lignocellulosic material, but addition of alkali (hydroxyl ions) stopped the repolymerization reaction and led to permanent lignin degradation. The present experimental conditions were analogous to this, as 2% sodium hydroxide had been present in the bed during autoclaving. Hence under the conditions of high temperature, pressure, and acidity prevailing during autoclaving, there could be a possibility of liberation (degradation) of lignin polymer (from the mean value 27.79%, of the lignin fraction, given in Table 1). Thus, some of the degraded inhibitory phenolic compounds from the pine needle bed (as predicted in FT-IR spectra), might have played a role to some extent, in inhibiting the growth and production of all the strains of Lactobacilli used in the present studies. Despite the presence of inhibitory compounds, the *Lactobacillus* strains showed reasonably good growth (observed in Fig. 4: 4(b), 4(c), 4(d), 4(e), and 4(f)) and lactic acid production. This may be attributed to the presence of vitamins A and C in needles or nitrogenous compounds and vitamins (from whey), which are required by the Lactobacilli as growth activators. Lucchini et al. (1990) reported that phenolic compounds such as phenyl ethyl alcohol and benzyl alcohol showed inhibitory, lethal, or membrane-altering properties due to their lipophilic nature, especially in gram negative bacteria (having lipo-polysaccharides). Being gram positive bacteria, Lactobacilli have lesser chances to be affected by alcohols, as they possess a thicker peptidoglycan layer in cell walls, without any exposed lipo-polysaccharides. Alcohols are known to affect the membrane lipids through their dissolving action, which affects the membrane fluidity. The microorganisms resist or acclimatize themselves in the presence of ethanol through change in their membrane lipid composition, which restricts the entry of ethanol, and the cellular leakage is minimized due to enhancement in hydrophobic barriers (Ingram 1986). Other Lactobacilli, such as *L. homohiochii* and *L. heterohiochii*, have been found to be highly alcohol resistant due to increase in ratio of unsaturated to saturated fatty acid (Ingram and Buttke 1984). This high tolerance to alcohol is associated with the presence of very long C20-C30 saturated monoenoic acids in high amounts (Shmidt et al. 1986). Nowak and Libudzisz (2006) found no effect of phenol or cresol on the growth or survival of intestinal lactic acid bacteria at 2, 20, and 100 micro g/mL doses. Hence the resistance of the present *Lactobacillus* strains towards some of the possible phenolic compounds from pine needle bed could be understood.

Scanning Electron Micrographs of Bacterial Growth on the Solid Bed

The SEM micrographs in Fig. 5: 5(a), 5(b), 5(c), 5(d), and 5(e), exhibited clusters of Lactobacilli strains-1,2, 3, 4, and co-coculture, fused growth and some distinct capsular rod-shaped bacterial cells, while others formed aggregates adhering with the bed material at 120 g/L whey-substituted glucose media application (under 5000X magnification). Figure 5(c) for strain-3, again shows lesser cell population, hence it provided the least lactic acid production of 18.02 g/L mean value (mentioned in Table 4). Figure 5(e) for co-culture indicated dense growth of bacteria very closely aggregated to each other

provided the maximum lactic acid production of 44.86 g/L mean value (mentioned in Table 4). The aggregates as well as separate rod shaped Lactobacilli cells adhering to the bed material can be observed in the SEM micrographs in Fig. 5. This clearly indicates that the present bacterial strains bear the capacity to grow on the bed material despite the possibility of the release of inhibitory phenolic substances from the pine needle bed material during autoclaving.

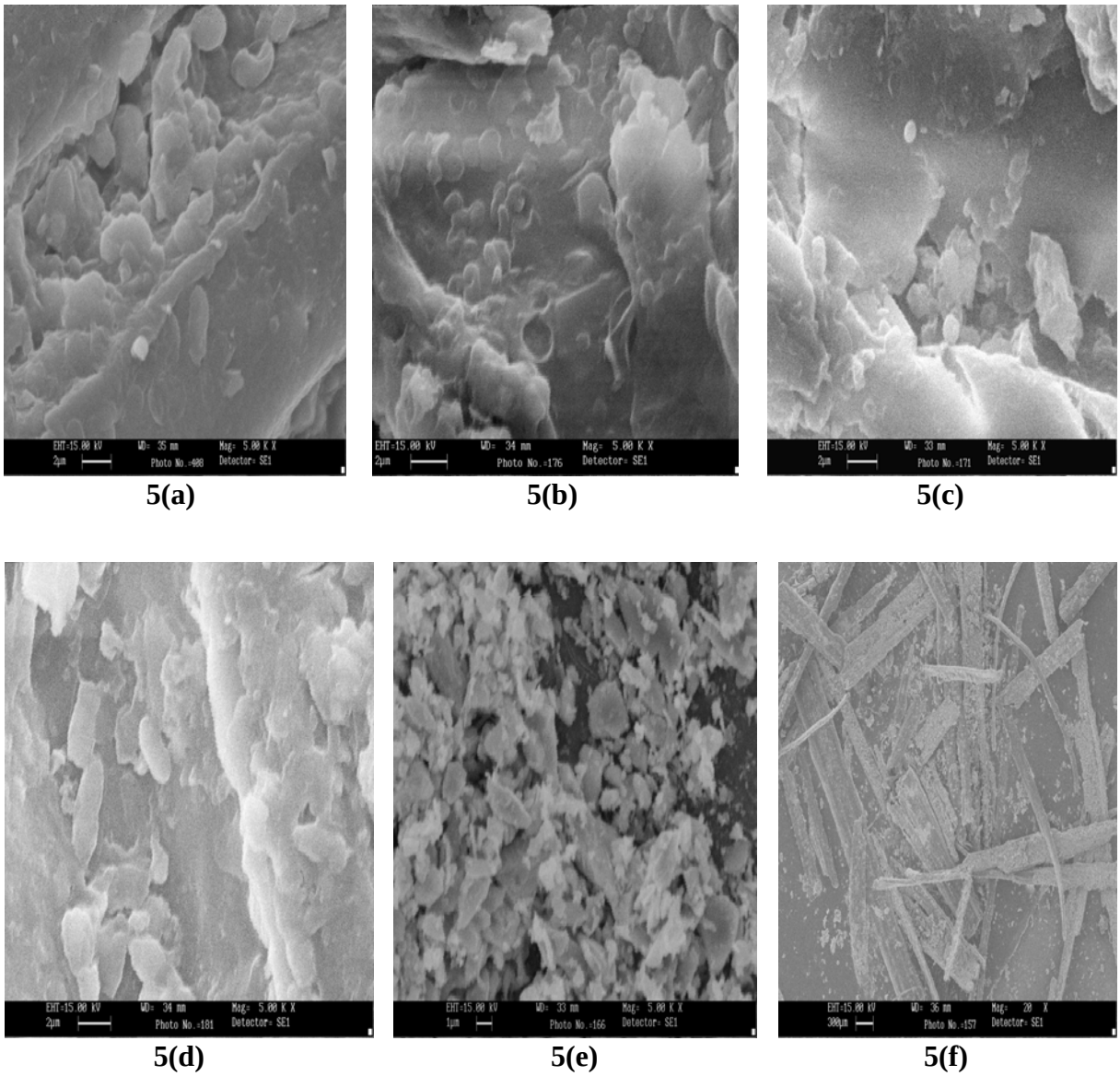

Fig. 5. SEM micrographs showing the growth of Lactobacilli on solid bed of pine needles. Frames 5(a), 5(b), 5(c), 5(d), and 5(e) show the growth of the strains-1, 2, 3, 4, and coculture, respectively, at 5000X magnification, on pine needle bed fed with a production media containing whey lactose (50 g/L), combined with glucose (70 g/L) at 120 g/L total sugar level, after six days incubation, while 5(f) shows pine needles in bed at 20X magnification.

FT-IR Studies of Pine Needles

The FT-IR analysis of a dried ground pine needle bed sample shown in Fig. 6 indicated peaks corresponding to different functional groups associated with various compounds predicted to be present in the pine needle bed material. These compounds could be liberated by degradation reactions, under high temperature and mild acidic conditions prevalent during autoclaving, and thereby affect the lactic acid producing bacterial strains under study.

The peak around 1378 cm^{-1} could be attributed to C-H bending vibrations in cellulose and hemicellulose, while the peak near 1165 signifies glycosidic linkages and showed C-O, C-O-C, stretching, and C-OH bending vibrations in arabinoxylans of hemicelluloses (Kumar et al. 2010b). The peak detected at 2921 cm^{-1} indicates a symmetric C-H stretching vibration. The spectral region near peak observed at 1730 cm^{-1} suggests C=O stretching vibrations due to presence of carboxyl group (Chauhan et. al 2008). The region detected around absorption band near 3420 to 3530 cm^{-1} indicated O-H stretching vibrations in guaiacyl and syringyl rings (which form monomer units in lignin) under intramolecular hydrogen bonding, while the peak near 1326 cm^{-1} may be attributed to C-O stretching of phenol (Kumar et al. 2010b). The absorption peak around 3413 cm^{-1} indicates the presence of O-H stretching of phenolic hydroxyl group.

The FTIR studies of the dry pine needles indicated about three peaks within the range 3420 to 3530 cm^{-1}, as shown in Fig. 6, out of which only one remained near 3429 cm^{-1} after autoclaving, while the other two disappeared. This clearly suggests the degradation of lignin in the bed material and release of phenolic compounds (from the bed material during the autoclaving process) that might have affected the growth and production of the present lactic acid producing strains.

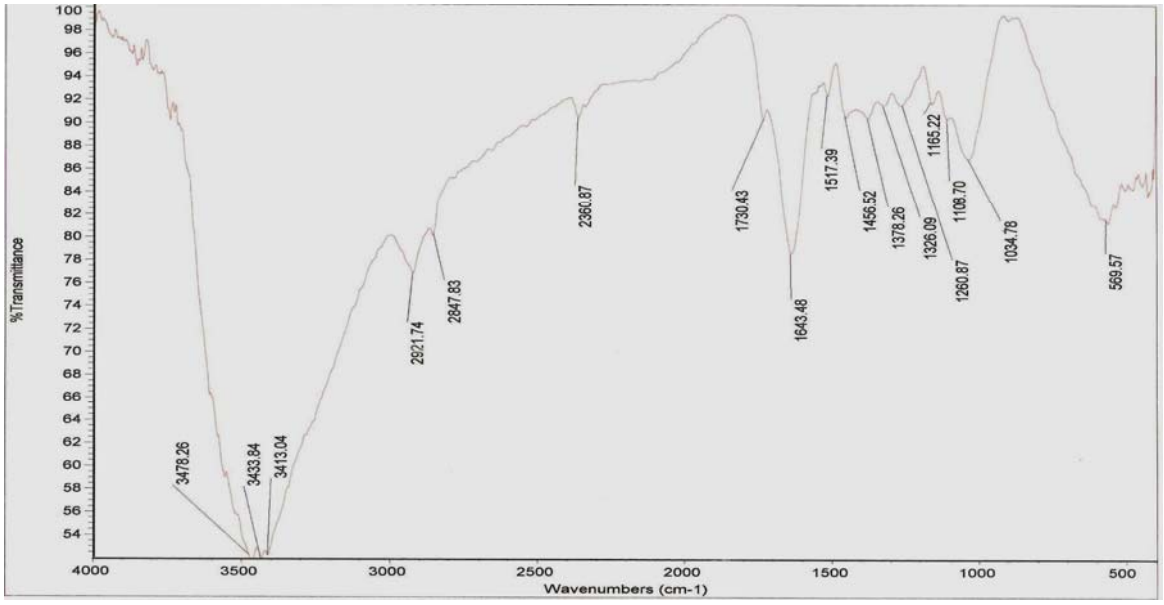

Fig. 6. FT-IR spectra of a ground pine needle bed sample in dried state

CONCLUSIONS

The present investigation provides a novel approach for the production of lactic acid by Lactobacilli through solid state fermentation using a bed of ground pine needles supplied with appropriate media which supports the growth and production of lactic acid. Use of 80 g/L pure glucose and 120 g/L whey-substituted glucose provided maximum individual lactic acid production in the first and second set of experiments, respectively. The co-culture exhibited the highest production in each set of experiments, while strain-3 showed the lowest, in both the media. The co-culture proved to be better than its constituent strains-1 and 2. The whey substitution effectively supported the growth and production of the bacterial strains. In the whey-substituted media, the nitrogenous and vitamin components from whey stimulated all the strains for higher growth and production.

Ability to utilize pentose (from bed), use of other lactose transporters, vitamins from pine needle bed, proteins, and vitamins in whey (in case of whey substituted, glucose) may also have played their role in enhancing the lactic acid production, despite the inhibition in lactose uptake by glucose, through the *lac* operon and presence of some of the phenolic compounds from the bed material.

The *Lactobacillus* strains are quite resistant to some of the phenolic substances that are possibly liberated from the bed material during autoclaving. The role of inexpensive pine needles in supporting the solid state fermentative production of lactic acid with Lactobacilli in presence of pure sugars and abundantly available waste material such as whey, may be investigated further to minimize environmental pollution due to forest fires and for possible production of valuable biochemical products.

On an industrial scale, solid-state production, using larger bioreactors such as multiple tray type, rotary drum type, may be more effective in enhancing the utilization of ground pine needles biomass and liquid waste such as whey for solid state lactic acid production.

Further studies are required regarding the utilization of used fermentation bed containing residual sugars, acids, live bacterial cells, and possibly some inhibitory phenolic compounds, in anaerobic digestion with appropriate phenol degrading and carbohydrate utilizing cultures. This may provide biogas in addition to production of lactic acid. Design of appropriate reactors and application of recombinant cultures and appropriate pretreatments of the pine needle bed material for solid state fermentation process should also be investigated in future.

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