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Characterization of Lactate Dehydrogenase Enzyme Immobilized to Magnetic Nanoparticles

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Abstract.- Magnetic nanoparticles (Fe_3O_4) were synthesized by co-precipitating Fe^{+2} and Fe^{+3} ions in an ammonia solution and treating under hydrothermal conditions. Lactate dehydrogenase (LDH) was covalently bound onto Fe_3O_4 magnetic nanoparticles via carbodiimide activation. The binding of LDH to magnetic particles was confirmed by fourier transform infrared (FTIR) spectroscopy analysis. The conjugated peaks were obtained at 1304.2cm^{-1} and 1075.2cm^{-1}. The binding efficiency was calculated 75-85% independent of the amount of particles used. The bound LDH exhibited significantly improved activity, stability and efficiency when compared with free LDH enzyme. Furthermore, the immobilized enzyme showed a better tolerance to temperature and pH variations. The optimal enzyme activity of both immobilized and free at 37ºC remains unchanged. The optimum pH for both free and immobilized LDH enzyme remains same. The storage stability of the immobilized enzyme is enhanced four fold to the stability of the free enzyme. These studies reveal that the improvements have been observed in the functionality of immobilized LDH enzyme that were perhaps due to structural and conformational changes of the bound enzyme that prevented auto-digestion and thermal inactivity. Hence the immobilized LDH enzyme will contribute to its better uses in various biological and clinical applications.

Key words: lactate dehydrogenase, magnetic particles, immobilization.

INTRODUCTION

The magnetic nanoparticles are widely used for the immobilization of proteins, enzymes, immunoglobulins and other biologically active molecules (Halling and Dunnil, 1980; Arica *et al.,* 2000; Akgol *et al.,* 2001, Chen and Su, 2001; Horak *et al*., 2001; Josephson *et al*., 1999; Sauzedde *et al*., 2000; Schutt *et al*., 1997). The magnetic nanoparticles are single domain particles with discrete randomly oriented magnetic moments. When placed in an external magnetic field their moments rapidly rotate into the direction of the magnetic field and enhance the magnetic flux. When the external magnetic field is removed brownian motion is sufficient to cause the moments and particles themselves to randomize and have no magnetic remanence (Riew *et al*., 1976). Because of their specific shape, size (diameter 5-100 nm) and surface chemistry the magnetic particles have numerous applications such as drug delivery (Schutt *et al*., 1997; Rudge *et al*., 2000; Lubbe *et al*., 2001) cell engineering, tissue repair (Josephson *et al*., 2001; Katz *et al*., 2002) and/or diagnostics (Gupta and Hung, 1989; Sahoo and Labhasetwar, 2003).

The magnetic nanoparticles as support have several advantages such as a) controllable size up to tens of nanometers, which is close to the biological entity of interest (Grady, 2003); b) can be coated with biological molecules to interact with or bind to a biological entity; c) can remotely position biological materials using an external magnetic field; d) can generate inductive heating effect by applying an external alternating magnetic field; and e) can be used as drug delivery system because of its strong magnetic property and low toxicity (Huang *et al*., 2003). The magnetic nanoparticles called magnetite are comprised of Fe^{2+} and Fe^{3+} ions in a 1:2 molar ratio, $FeO{\cdot}Fe_2O_3$, where half of the Fe^{3+} ions are tetrahedrally coordinated and the other half are octahedrally coordinated whereas all the Fe^{2+} are octahedrally coordinated (Mackinnon *et al*., 2003).

Enzyme immobilization onto insoluble support is a desired biological procedure because of its possible applications in product purification and

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catalyst recycling (Jia *et al*., 2003). Furthermore, immobilization provides many advantages such as: 1) enhanced stability, 2) easy separation of product from reaction mixture, 3) possible modulation of the catalytic properties, 4) easier prevention of microbial growth (Bornscheuer, 2003). In the last decade, magnetic nanoparticles have been widely used as support for this purpose, because of their unique properties (Huang *et al*., 2003; Liao and Chen, 2001; Koneracka *et al*., 1999; Kondo and Fukuda, 1997). Binding of enzymes can be done directly via carbodiimide activation. The carbodiimide is considerably promising because of its simplicity, efficiency and long term stability (Huang *et al*., 2003).

It has recently been reported by many scientific groups that enzymes (yeast alcohol dehydrogenase, lipase and cholesterol oxidase) after immobilization to the magnetic nanoparticles retain catalytic activity without any loss (Huang *et al*., 2003; Liao and Chen, 2001; Gilles *et al*., 2005).

Vertebrate lactate dehydrogenase (LDH) is a tetramer enzyme found either as H- type or as M-type and exists in the form of five isozymes (LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5) (Basaglia, 1989; Fritz and Jacobson, 1965). These isozymes differ in various physicochemical, immunological and physiological properties (Javed and Waqar, 1993; Brooks, 2000).The LDH-4 and LDH-5 isozymes are mainly found in the skeletal muscles where anaerobic glycolysis predominates whereas LDH-1 and LDH-2 are mainly found in heart muscles where pyruvate is oxidized via Krebs cycle (Zubay, 1993). LDH is clinically important for its applications in coupled enzyme assays such as determination of ATPase, myokinase and pyruvate kinase activities (Penesfsky and Bruist, 1984; Brolin, 1983; Fujii and Miwa, 1983; Beutler, 1971). LDH enzyme has also some clinical importance because serum level of its certain isozymes reflects pathological condition in particular tissues. This enzyme is also used in the determination of lactate, pyruvate and various other metabolites (Noll, 1984; Lamprecht and Heinz, 1984).

In this study the immobilization of lactate dehydrogenase (LDH) enzyme from rabbit skeletal muscle to magnetite is made by using carbodiimde method (Huang *et al*., 2003). The binding of lactate dehydrogenase to magnetic nanoparticle was confirmed by Fourier transform infrared (FTIR) spectroscopy. The stability and activity of bound lactate dehydrogenase was also determined.

MATERIALS AND METHODS

Materials

Acrylamide, bis-acrylamide, ammonium per sulphate (APS), sodium dodecyl sulphate (SDS), glycine, ethanol, carbodiimide-HCl [1-ethyl-3-(3-dimethyl-aminopropyl)], ammonium hydroxide, bovine serum albumin (BSA), iron (II) chloride tetrahydrate 97% ($FeCl_2.4H_2O$), iron (III) chloride hexahydrate 99% ($FeCl_3.6H_2O$), HCl, NaOH, bradford reagent, sodium dihydogen phosphate, disodium hydogen phosphate Trizma base and NaCl were purchased from Sigma- Aldrich, USA. lactate dehydrogenase (LDH) from rabbit muscle (EC 1.1.1.27) was purchased from Behring, Germany. Protein marker (PageRuler^{TM} Protein Ladder # SM0661) was purchased from Fermentas, USA. Lactate Dehydrogenase assay kit was purchased from Biocon, Germany.

SDS-PAGE analysis of LDH

Reducing and non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to check the homogenety of lactate dehydrogenase enzyme as described by Laemmli (1970). The protein concentration was determined by Bradford reagent assay using bovine albumin serum (Bradford, 1976) as standard.

Determination of LDH activity

The activity of lactate dehydrogenase was determined by using BIOCON lactate dehydrogenase kit. To perform macro-assay 10µl of LDH solution (1mg/ml in PBS) was added into 2500µl of working reagent taken in the cuvette. After gentle mixing, absorbance was read at 340nm. The absorption decrease per minute was noted.

Preparation of magnetic nanoparticles

The magnetic nanoparticles were prepared using methods described by Huang *et al*. (2003) and Gilles *et al*. (2005) given as follows: Iron (II) chloride and iron (III) chloride were dissolved in double distilled water in ratio 1:2 at the final

concentration of 0.25M iron ions. It was chemically co-precipitated at room temperature (25ºC) by adding NH_4OH solution (30%) to maintain the pH 10.0. The suspensions were heated at 80ºC for 35 minutes under continuous stirring. It was separated by centrifuging several times in water (25ml /wash) and then finally in ethanol at 2800 rpm, for 10 minutes at 25ºC. As a result of purification step impurities were removed from magnetic (Fe_3O_4) nanoparticles. The particles were finally dried in vacuum oven at 70ºC overnight. The dried particles showed attraction towards magnetic rod.

Attachment of LDH onto magnetic nanoparticles

The rabbit skeletal muscle LDH was attached to the magnetic nanoparticles by the method described by Liao and Chen (2001), Gilles *et al*. (2005) and Li *et al*. (2003) as follows: 50mg of magnetic nanoparticles was added to 1ml of phosphate buffer (0.05M, pH 7.4). The mixture was sonicated for 15 minutes after adding 0.5ml of carbodiimide solution (0.02g/ml in phosphate buffer, 0.05M pH 7.4). Following the carbodiimide activation, 2ml of lactate dehydrogenase (0.25mg/ml in phosphate buffer) was added and the reaction mixture was sonicated for 30 minutes in a sonication bath at 4ºC. The mixture was centrifuged at 3000 rpm for 10 minutes. The isolated supernatant was stored in an eppendorf tube at -20ºC. The precipitates containing Fe_3O_4 nanoparticles and Fe_3O_4 bound LDH (Fe_3O_4-LDH) were washed three times with phosphate buffer (0.05M, pH 7.4), 0.1M Tris-Cl (pH 8) and finally with 0.1M NaCl respectively and then used for stability and activity measurements. NaCl was added to enhance the separation of magnetic nanoparticles. The immobilized enzyme was stored at -20ºC for further use.

Determination of immobilization efficiency

The amount of protein (concentration) in the supernatant was determined by Bradford assay as described by Gilles *et al*., 2005. The amount of bound LDH was determined by using the formula: $A = (Ci-Cs)\ V$.

Where A is the amount of bound LDH, Ci and Cs is the concentration of LDH initially added for attachment, and in the supernatant, respectively (mg/ml), V is the volume of the reaction medium (ml).

Fourier transform infrared (FTIR) spectroscopy analysis

Samples (LDH, Fe_3O_4 and LDH-Fe_3O_4) were homogenized individually in nujol for 15 minutes, using teflon mortar and pestle. Few drops of sample were taken on sodium chloride cell cleaned with carbon tetrachloride and their FTIR spectra were taken via FTIR Spectrometer (Perkin Elmer).

Effect of temperature on LDH

The effect of temperature on the activity of free and immobilized LDH was examined by measuring its relative activity at 340 nm after incubating reaction mixture at various temperatures (37ºC, 50ºC, 60ºC). The absorption decrease per minute was noted and its percentage relative activity was determined by using the formula: $\Delta A/min \times 4127 \times 50/100$

Effect of pH on LDH

The effect of pH on the relative activity of free and immobilized LDH was examined in the pH range of 6 to 9 at 37ºC. At the desired pH, percentage relative activity was determined by Biocon LDH kit assay.

Effect of storage on stability of LDH

The storage activity of free and immobilized LDH was determined by storing it at 25ºC for 30 days. The residual activities were assayed after five days of time interval.

RESULTS AND DISCUSSION

Activity of free LDH

The enzymatic activity of LDH (used for immobilization) was determined by Biocon kit assay which was simple and relatively efficient method. In the reaction mixture the coenzyme NADH strongly absorbs light at 340nm and hence the reaction rate was determined from the disappearance of NADH over a time period. The oxidation of NADH was equimolar to the formation of lactate which is determined by spectrometric analysis and calculated as 578 U/mg.

Determination of immobilization efficiency

The unbound LDH was determined by assaying the protein content in the supernatant. The percentage of LDH bound was 75 to 85% irrespective of the amount of nanoparticles. The quantity of the nanoparticles were used to its saturation but any increase in the binding efficiency was not obtained which is not in agreement with studies reported by Gilles *et al*. (2005) and Huang *et al*. (2003). The low binding efficiency may be attributed to the structure and binding mechanism of the enzymes.

Binding confirmation

The binding of LDH enzyme to magnetic nanoparticles was examined by Fourier transform infrared (FTIR) spectroscopy analysis. The spectra of pure LDH, naked Fe_3O_4 and LDH-bound Fe_3O_4 is shown in Figure 1 (A, B and C). The characteristic bands of nujol (CH_3, CH_2, CH) were observed in the spectra of LDH, Fe_3O_4 and Fe_3O_4-LDH as the samples were homogenized with it. The sample peaks of LDH and Fe_3O_4 were observed at 1153.8cm^{-1} and 1155.7cm^{-1} (Fig. 1A, 1B) respectively. However these peaks were not apparent in the spectra of Fe_3O_4-LDH (Fig. 1C). Rather new peaks were obtained at 1304.2cm^{-1} and 1075.2 cm^{-1} which confirms the binding of LDH to the nanoparticles. The binding of LDH to nanoparticles suggests the involvement of -NH2 and -COOH group after activation with carbodiimide. These results are in agreement with the studies reported by Gilles *et al.* (2005) and Huang *et al.* (2003).

Physiochemical effects on LDH

The effect of temperature on the activity of free and bound LDH was examined by measuring its relative activity when incubated at various temperatures (Fig. 2). At 37ºC the activity of free and bound enzyme remains unchanged for 30 minutes. At 50ºC the activity of both the (free and bound) enzymes was reduced to 30% and 40% after 30 minutes, respectively. At 60ºC the activity of free and bound enzyme showed drastic decrease to 12% and 18% after 30 minutes respectively. The decrease in activity is attributed to a dramatic conformational change in the structure of LDH perhaps because of

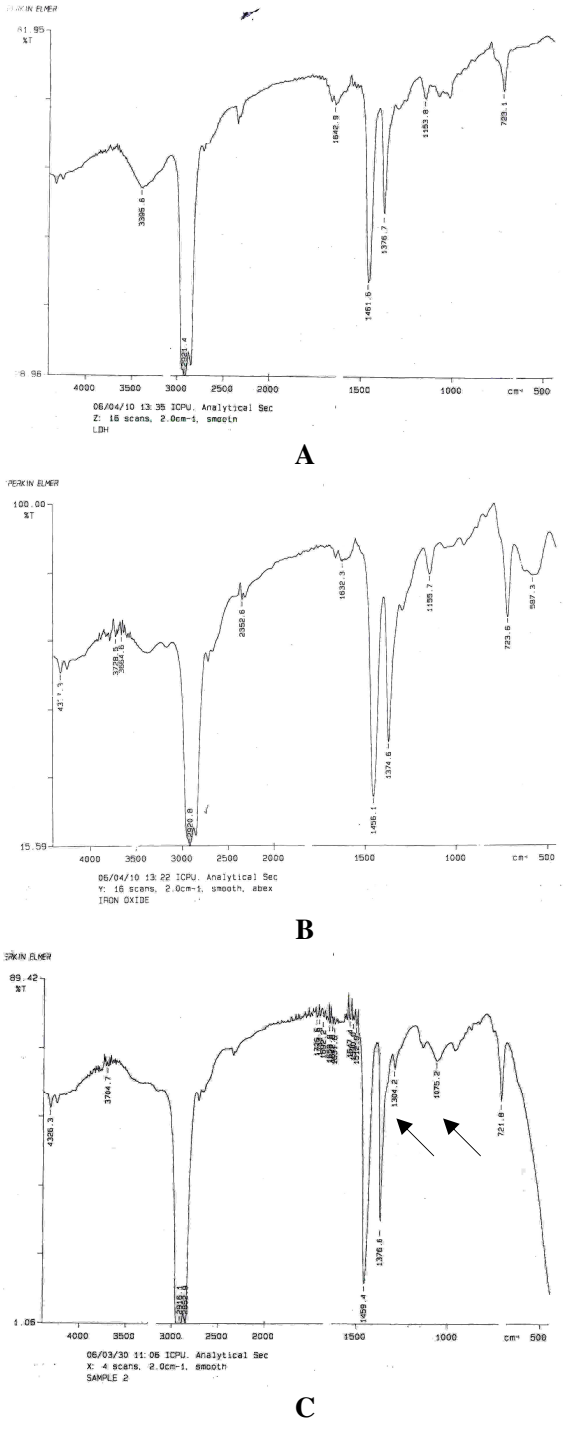

Fig. 1. A, FTIR spectra of lactate dehydrogenas; B, FTIR spectra of Fe_3O_4 magnetic nanoparticles; C, FTIR spectra of lactate dehydrogenase-Fe_3O_4

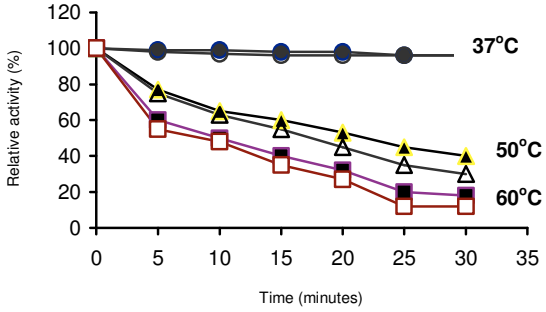

Fig. 2. Effect of temperature on the activity of free (○-○, Δ-Δ, □-□) and bound to magnetite (●-●, ▲-▲, ■-■) LDH at pH 7.5.

the denaturation of the enzyme. The conjugated LDH tolerated temperatures for relatively more time due to perhaps its better orientation.

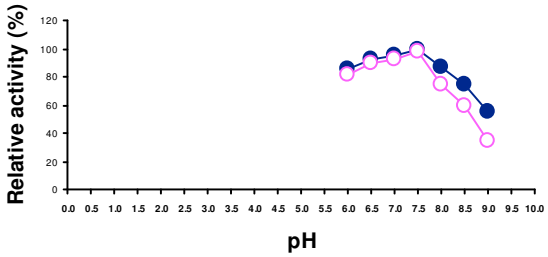

Fig. 3. Effect of pH on free (○-○) and immobilized (●-●) lactate dehydrogenase enzyme at 37°C.

The effect of pH on the activities of the free and bound LDH was determined in the pH range of 6 to 9 at 37ºC (Fig. 3). In the pH range between 6 and 7 the activities of the free and bound LDH were quite similar and reached to a maximum at pH 7.5. The activity then decreased from 7.5 to 9. In this range, the activity of the bound LDH was comparatively higher than its free counterpart. The bound enzyme showed better tolerance to the variation of solution pH. The enzyme activities of LDH (free and bound) in the pH range of 6 to 7 did not suffer any major activity constraint. The decrease in activity observed at pH higher than 7.5 is indicative of constraints with increase in pH.

The storage stability of free and bound LDH was determined for 30 days (Fig. 4). However, the residual activity of bound LDH was comparatively higher during the same time period. It obviously revealed that the storage stability of LDH was improved significantly after being bound to Fe_3O_4 nanoparticles. This considerable enhancement of stability was due to LDH proper fixation on the surface of magnetic nanoparticles.

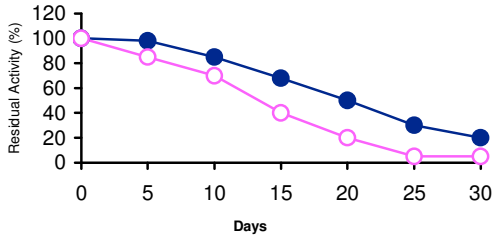

Fig. 4. Effect of storage on stability of free (○-○) and immobilized (●-●) lactate dehydrogenase at pH 7.5

This work explores a simple and novel method to immobilize LDH to magnetic nanoparticles which can be used for diagnostic purposes in coupled enzyme assays in myocardial infarction and other pathological diseases.

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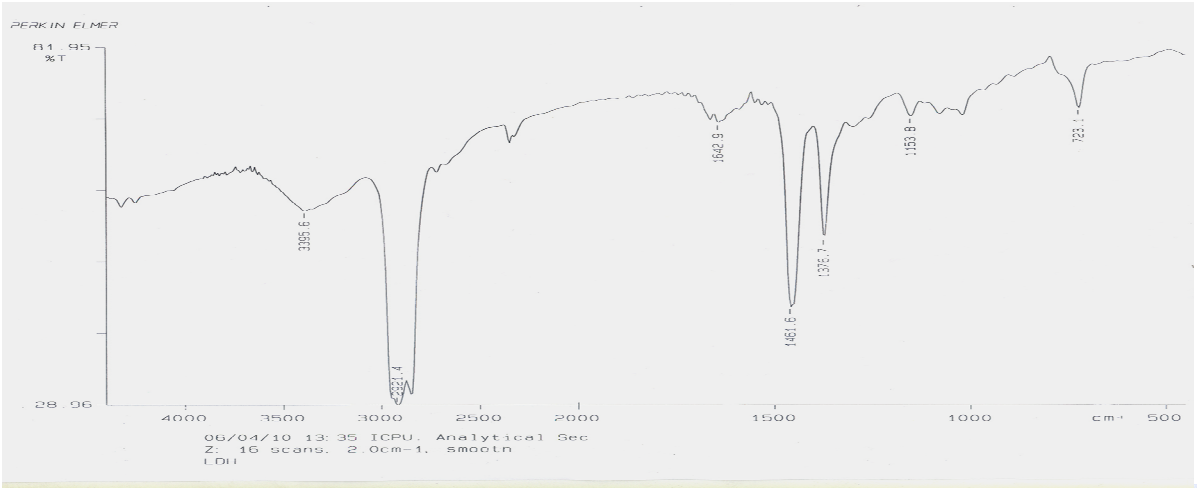
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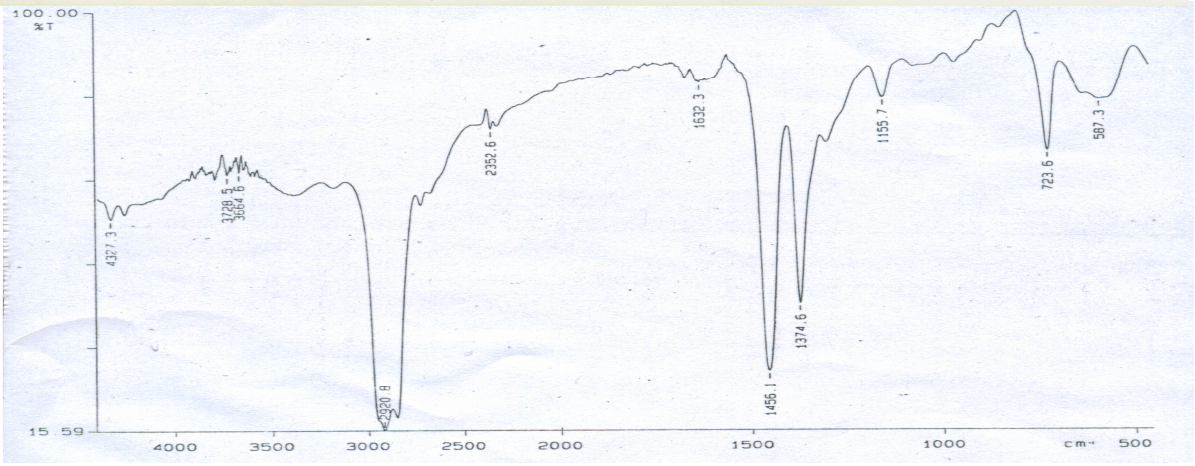
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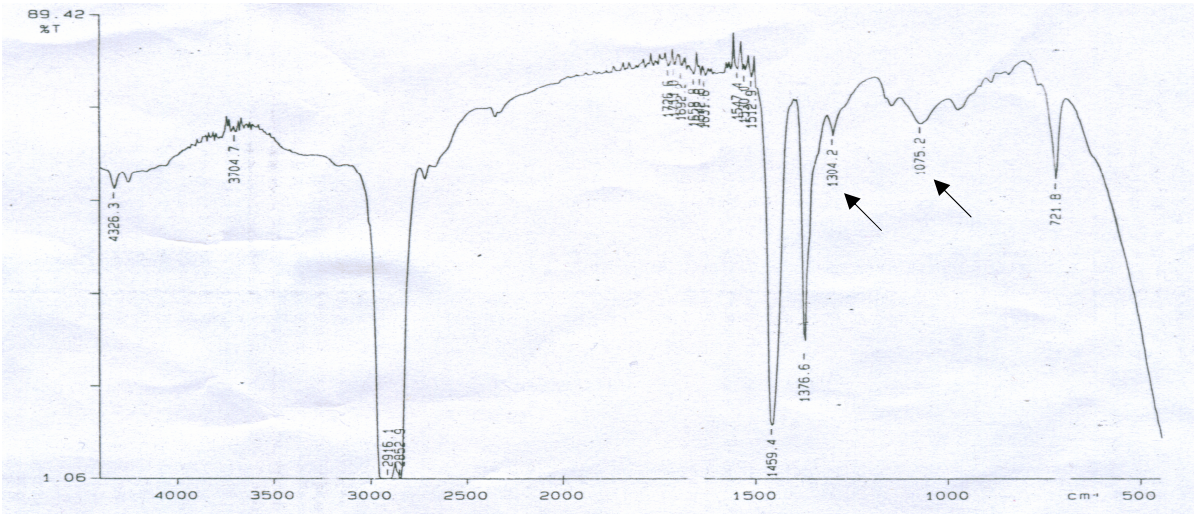
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