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Biotransformation of succinodinitrile into corresponding high value compounds using nitrile hydrolysing enzyme from *Microbacterium paraoxydans* RS15

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Abstract

A Gram-negative bacterial strain, identified as *Microbacterium paraoxydans* RS15 using 16S rRNA technique, has been used in the present study. Total 40 cultures screened through primary screening were further selected for secondary screening in shake flask containing MSM media and nitrile substrate. This strain was grown on a variety of aliphatic and aromatic nitriles (benzonitrile, Mandelonitrile, Propionitrile). This strain was grown well successfully on Succinodinitrile containing plates from 10mM to 200mM concentration. This isolate showed growth associated nitrile hydrolysing enzyme production after 11 hours in bioreactor and 24 hours of incubation in shake flask at optimal conditions. Enzyme activity increased to 1109 and 687 U/ml/mg when the media was supplemented with Glucose and Sodium Citrate respectively. Scale up of enzyme production was studied in 5L *in-situ* bioreactor. Bacterial cells when exposed to different solvents like Toluene, Hexane and Ethyl acetate showed a significant retention in nitrile hydrolysing enzyme activity and the maximum was in hexane. The kinetic characterization of the nitrile hydrolysing enzyme exhibited temperature optima at 30°C and pH 7. Biotransformation of succinodinitrile into corresponding high value compounds like Succinic acid and amide using nitrile hydrolysing enzyme from *Microbacterium paraoxydans* was carried out and the same was authenticated using analytical technique GC-MS.

Key words : Biotransformation, in-situ bioreactor, *Microbacterium paraoxydans, nitrile hydrolysing* enzyme.

INTRODUCTION

Nitriles are organo-cyanides (R-CN) which are widely spread in the environment due to natural sources (plants, bacteria) as well as from industrial processes[1]. They are in various forms such as cyanoglycosides (mostly), ricinine, phenylacetonitrile and cyanolipids[2]. The situation has been aggravated by the surfeit use of nitriles in chemical industries[3], ranging from solvents (e.g. acetonitrile), polymers (e.g. adiponitrile and acrylonitrile) to fine chemical syntheses (e.g. enantiopure carboxylic acids and amides)[4]. The nitriles are toxic, carcinogenic and mutagenic[2]. Microorganisms can assimilate them as carbon and/or nitrogen sources by means of the role of nitrile hydrolysing enzymes[5]. Biotransformations of nitriles proceed by two metabolic pathways; Nitrile hydratase (NHase) catalyses the hydration of a nitrile to an amide, followed by its conversion to an acid and an ammonium by amidase, whereas nitrile hydrolysing enzyme catalyzes the hydrolysis of a nitrile directly to an acid and an ammonium [6].

Nitrile hydrolysing enzyme are α/β hydrolases (EC 3.5.5.1; 3.5.5.2; 3.5.5.4-3.5.5.7). Various nitrile hydrolysing enzymes isolated from bacteria, fungi and plant have been described. Their application in the production of acrylic acid, β-alanine, *p*-methoxyphenylacetic acid [7-8] have been reported. The range of possible nitrile hydrolysing enzyme applications has been recently broadened but in most cases the parameters of the reactions need to be improved to establish viable industrial processes. To achieve this goal, several methods have been used, primarily in screening for enzymes from new sources [9], medium engineering, and variation in process parameters[10]. The novelty of work lies with the isolation of a *Microbacterium sp.* to degrade succinodinitrile into Succinic acid by biological way.

MATERIALS AND METHODS

Chemicals and Composition

Succinodinitrile and Succinic acid used in the present study were obtained from Merck Specialities Pvt. Ltd. (Mumbai, India). All reagents were of analytical grade. The Minimal Salt Media (MSM) contained following salts procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), (per litre of media): $Na_2HPO_4.2H_2O$ (2g), KH_2PO_4 (2g), $MgCl_2.6H_2O$ (0.1g), NH_4Cl (0.1g). Nitriles were added aseptically after autoclaving.

Primary screening of Isolates on solid plates with substrate

Isolates were available previously in the lab, isolated from industrial waste using stringent enrichment technique. Isolates were screened on solid plates with Succinodinitrile as substrate from 10 mM to 200 mM concentration in Minimal salt media (MSM) at 30°C for 24 hours.

Bacterial Growth in shake flask

The bacterial isolates were isolated on MSM + nitrile substrate plate, where nitrile substrate acts as carbon and nitrogen source. The isolate was then maintained on Nutrient agar plates. MSM media and MSM supplemented with 100mM succinodinitrile plates were used to grow the bacterial strain. For the production of the enzyme, a single colony from freshly streaked NA plate of culture, was inoculated in seed medium i.e NB. At an absorbance between 0.4 - 0.6 at 600nm, one ml (2%) of the inoculum was transferred from the seed medium to the production medium (MSM) having 25mM substrate. The production medium was incubated at 30 °C in shaker at 180 rpm. Sampling was done at regular intervals. Absorbance of the sample was taken at 600nm, pelleting was done by centrifugation at 11,000 ×g for 10 minutes at 4 °C and the nitrile hydrolysing

enzyme activity of the cells was accessed. The same experiment was repeated with three more variations i.e. MSM +100mM substrate, MSM+ 25mM substrate + 25mM glucose, MSM + 25mM substrate + 25mM Sodium Citrate. Each of the experiment was done three times.

Scale-up in the Bioreactor

Scale up study was carried out in *in-situ* bioreactor (5 litres capacity) (Bioage Equipment and Services, Mohali, India) in MSM media supplemented with 25mM succinodinitrile/ 25mM succinonitrile + 25mM glucose. 3.5 L of media was inoculated with 2% inoculum. Fermentation conditions were 0.4 lpm, 150 rpm and 30°C. Sampling was done at regular intervals of 2hr and samples were processed further for intracellular enzyme activity. Absorbance of the sample was taken at 600nm and pelleting was done by centrifugation (Remi Sales and Engineering Limited, New Delhi, India) at 11,000× g for 10 minutes at 4°C.

Nitrile hydrolysing enzyme assay[11]

Pellet obtained was suspended in 0.2M phosphate buffer of pH 7. For 1 mg of pellet 40µl of buffer was added. Nitrile hydrolysing enzyme activity was assayed at 30°C for 15 min in a 60µl reaction mixture that contained 0.1M phosphate buffer, 100 mM Succinodinitrile and 20µl cell suspension. Reaction was stopped by adding 400µl 0.1 HCl and centrifuged at 10,000 rpm for 10 minutes at 4°C. Ammonia released during incubation was measured by Berthelot reaction (phenol-hypochlorite method), in which ammonia reacts with hypochlorite, to produce a stable blue complex (indophenol) at a λ of 640.

Calculation of enzyme activity:

The activity was then calculated based on standard formula: Net O.D/ (slope× time of the assay× pellet weight × enzyme added in the assay in ml)

The enzyme units were calculated as enzyme U/mg/ml/min.

Solvent stability

The cells were harvested after 6 hours from the bioreactor and after 24 hours from the shake flask. 50mg cells were suspended in 1 ml of 0.2M phosphate buffer. The absorbance of the cell suspension was noted at 600 nm and cell count was 1.6×10^9 cells per ml. 1ml of the cell suspension from each microcentrifuge tube was transferred to four different glass vials (Borosil Glassworks Ltd., India) containing four different solvents 1 ml each of Toluene, Hexane, Petroleum Benzine, Ethyl Acetate. One glass vial containing buffer suspended cells was the control of the experiment. These glass vials were then kept at shaker at 30°C and 180 rpm. The samples were taken for 6 hrs and after every hour, the sample was centrifuged at 11,000 ×g for 10 minutes at 4°C. Residual activity was determined by using the ammonia release assay[11].

Kinetic characterization

Temperature optimization

1mg of cells were taken in micro centrifuge tube and suspended in 40µl of 0.2M phosphate buffer of pH 7. Activity was determined at 6 different temperatures- 20° C, 25° C, 30° C, 35° C, 40° C, 45° C. The activity was recorded for each temperature and relative activities were calculated. The experiment was repeated three times and observations were recorded in graphical form.

pH optimization

1mg of cells suspended in 40µl of 0.2M phosphate buffer were taken to optimize pH with six variables 5, 6, 7, 8, 9 and 10. Enzyme assay was done and the relative activity was calculated at each pH.

Thermostability

20 mg cells were suspended in 1ml of 0.2M phosphate buffer and were incubated at 30°C and 40°C. After every one hour samples were taken and were centrifuged at 11,000×g at 4°C. Nitrile hydrolysing enzyme activity assay was performed by suspending the cell pellet in 0.2M, phosphate buffer of pH 7. The samples were taken up to 6hrs and the activity of the cells was recorded in U/mg/ml to represent it in graphical form.

Biotransformation

20mg cells dissolved in minimum quantity of phosphate buffer (0.2 M, pH 7) were suspended in hexane as organic solvent and 25 mM Succinodinitrile as substrate. The reaction was kept at 30°C and 180 rpm. Samples taken after every hour were dried by Rotary Evaporator (Yamato). The analysis of the biotransformed product was done by GC-MS.

Determination of transformation of Succinodinitrile to Succinic acid by GC-MS

Gas chromatography (GC) an Mass Spectrophotometry (MS) was performed on a Thermoscientific™ GC model name Trace 1300 and MS model name TSQ 8000 (triple quadrapole) gas chromatograph (Palo Alto, CA) by using a Thermo TG 5MS (30m X 0.25mm X 0.25µm column with helium as carrier gas at 1ml/min.

Method: The injector temperature was 250°C and the transfer line temperature was 300°C. The initial column temperature of 60°C was maintained for 2 min and then increased at the rate of 15°C/min to 250°C and hold it for 14 minutes. Injection volume was 1 µl. MS source temperature was 280 °C and run time was 27.78 min. High mass (m/z) cut-off was 350.

Table 1: Characterization and growth of RS15 on Succinodinitrile (SDN) after 24 hours

Culture	Growth on 10mM SDN	Growth on 25mM SDN	Growth on 100mM SDN	Growth on 200mM SDN	Gram stain	Morphology
RS15	+++	++	+ +	+	-v e	Light yellow, shining, opaque, regular margins, thin rods

RESULTS

Selection and Identification of a Nitrile-Utilizing Strain

During screening of the 14 bacterial strains with the substrates in our lab *Microbacterium paraoxydans* RS15 showed highest growth up to 200mM of Succinonitrile after 24 hours. Gram staining revealed it to be Gram ve, rod shaped bacteria, nonmotile and non-spore forming (Table 1). After primary screening growth profile of the organism was studied. The organism exhibited a lag phase of 4 hrs. The growth was linked with the decrease of succinodinitrile in the media. An excited general information led to study the nitrile hydrolysing enzyme activity of the organism and its identification of 16S rDNA, which revealed it to be 99.76% identical.

Shake flask experiments

Shake flask experiments with the RS15 were done to knew about the nitrile hydrolyzing enzyme production by method as per given above. 25mM succinodinitrile was used as substrate in the media. Absorbance was measured along with activity (U/ml/mg) of the intracellular enzyme at 600nm.

The maximum enzyme activity and absorbance reported were 507U/mg/ml and 0.1525 at 24 hours respectively (Fig. 1). The production was carried out till 47th hour. The activity started increasing after 21st hour and start decreasing after 41st hour. There was a good growth and activity between 20 to 41hours.

Growth and nitrile hydrolyzing enzyme production of RS15 in shake flask using MSM +100mM succinodinitrile as substrate.

After the good growth and activity at 25mM conc. of RS15, the isolate was grown at 100mM conc. of substrate. The maximum enzyme activity reported was 77U/mg/ml at 41 hours, while maximum absorbance was 0.136 at the same hour (Fig. 2). It infers that with increase in substrate concentration, growth of the bacteria was adversely effected and due to it, maxima was observed at 41st hour. The production was carried out till 63rd hour.

Effect of media supplements on Nitrile hydrolysing enzyme activity:

(i) Effect of glucose: In order to see the effects of growth enhancers or supplements such as sodium citrate and glucose on the growth and activity of nitrile hydrolyzing enzyme, these were added in the media along with substrate. Substrate and glucose concentration were kept as 25mM.

There was good activity and growth between 20 to 45 hours. The maximum enzyme activity reported was 1109U/mg/ml at 24 hours and the maximum absorbance was 0.5255 at 41hours (Fig. 3) which were quite 2 and 3.5 times respectively. This upsurge in the activity was quite appreciable with a little addition of glucose in. The production was carried out till 45th hour.

(ii) Effect of sodium citrate: Activity profile increased with the addition of sodium citrate as the maxima was at 687U/mg/ml at 24 hours and the maxima in the absorbance was 0.15 at 24th hour (Fig. 4).

Scale up in the Bioreactor

Growth and nitrile hydrolyzing enzyme production of RS15 in *in-situ* bioreactor using MSM+ 25mM succinodinitrile as

Table 2: Comparative analysis of shake flask and in-situ bioreactor

Parameters		Shake flask	*in-situ* bioreactor
Activity	w/o supplementation	507/24 hr	570/11hr
	Glucose	1109/24 hr	262/6hr
	Sodium citrate	687/24 hr	-
Absorbance	w/o supplementation	0.1525/ 24 hr	0.174/11 hr
	Glucose	0.5255/ 41 hr	0.663/7hr
	Sodium citrate	0.15/ 24hr	-

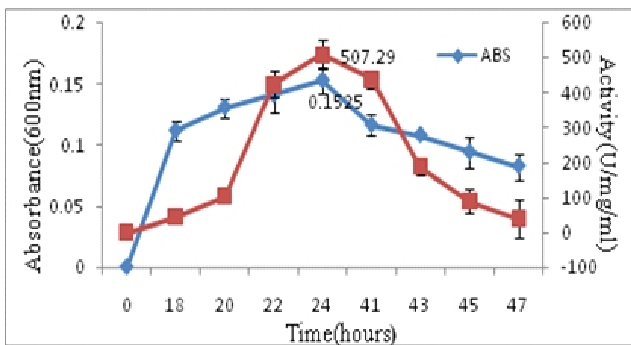

Fig 1: Activity profile of enzyme of RS15 in MSM + 25mM succinodinitrile showing the growth(blue) and activity(red) at 30 oC and 180 rpm.

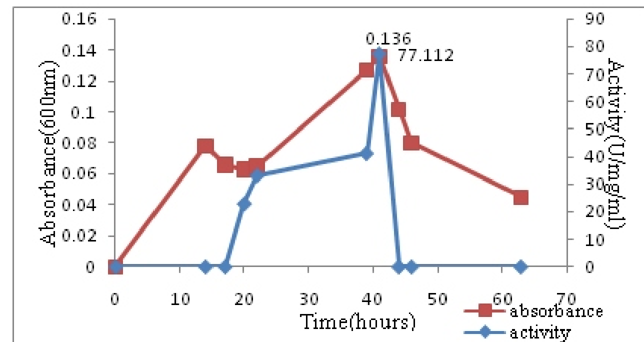

Fig 2: Activity profile of nitrile hydrolysing enzyme of RS15 in MSM + 100mM succinodinitrile showing the growth (red) and activity(blue) in shake flask at 30 oC and 180 rpm .

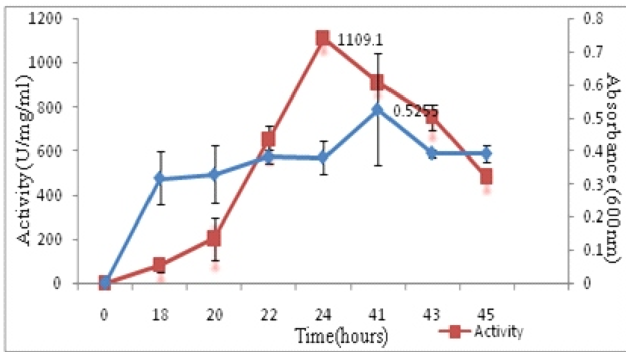

Fig 3: Activity profile of RS15 to show the effect of glucose in MSM + 25mM succinodinitrile + glucose showing growth(blue) and activity(red) in shake flask at 30 °C and 180 rpm .

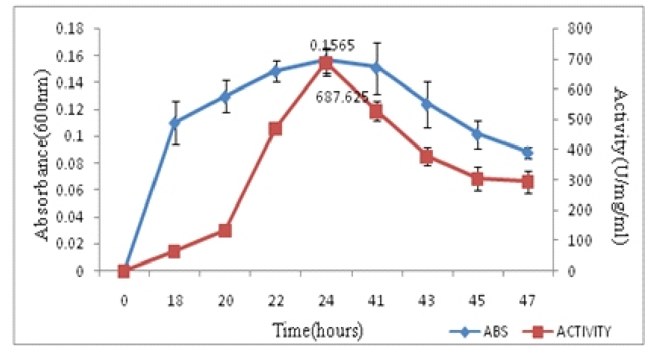

Fig 4: Activity profile of RS15 to show the effect of Sodium citrate in MSM + 25mM succinodinitrile + sodium citrate showing activity(red) and growth(blue) in shake flask at 30 °C and 180 rpm .

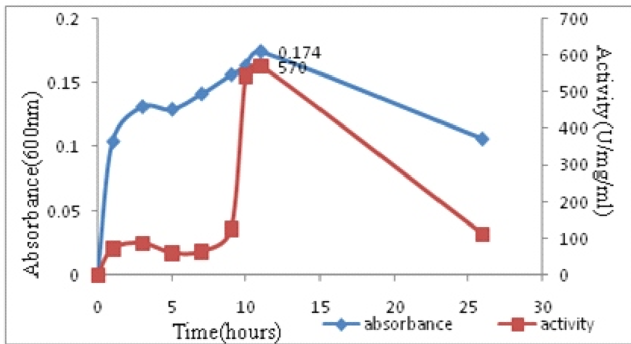

Fig 5: Activity profile of RS15 in 5L *in-situ* Bioreactor in MSM +25mM succinodinitrile showing growth(blue) and activity(red) at 30 °C and 180 rpm .

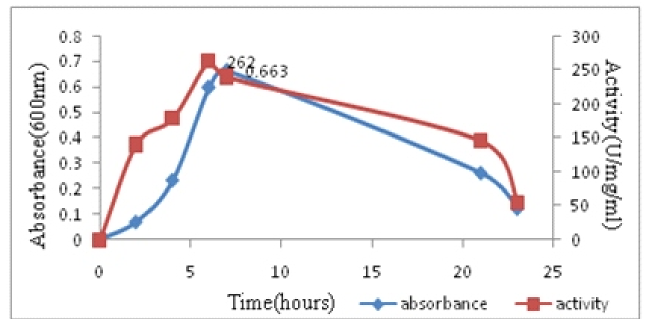

Fig 6: Activity profile of RS15 in 5L *in-situ* Bioreactor in MSM + 25mM glucose + 25mM succinodinitrile showing activity(red) and absorbance(blue) at 30 °C and 180 rpm .

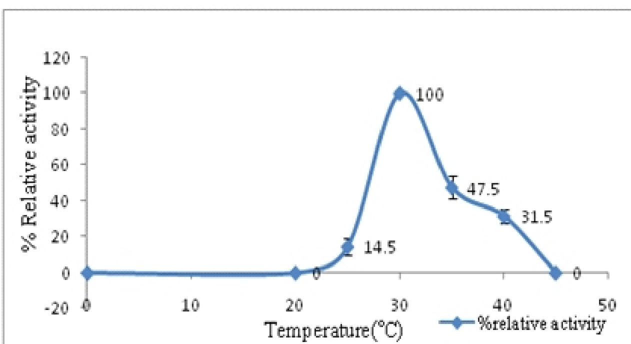

Fig 7: Temperature optima of nitrile hydrolyzing enzyme of RS15.

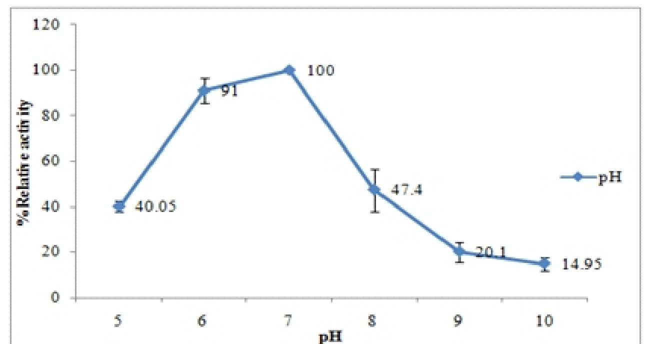

Fig 8: pH optima of nitrile hydrolyzing enzyme of RS15 at 30 °C.

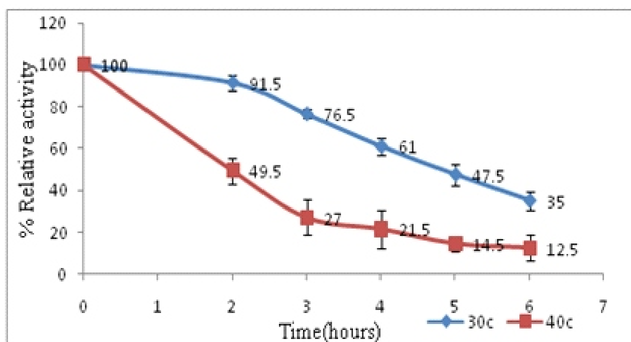

Fig 9: Thermal stability profile of nitrile hydrolyzing enzyme of RS15 at 30 °C and pH 7.

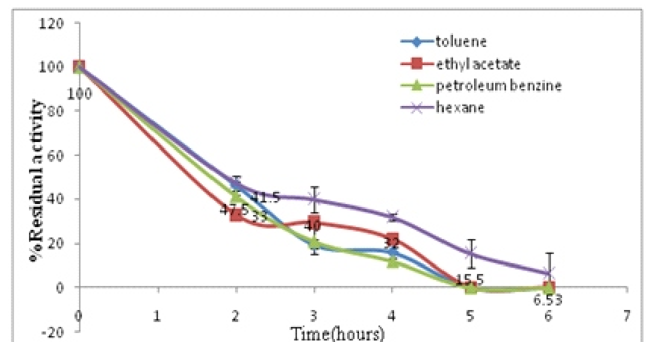

Fig 10: pEffect of different solvents on nitrile hydrolyzing enzyme activity at 30 °C; ethyl acetate(blue), toluene(red), petroleum ether(green) and hexane(purple).

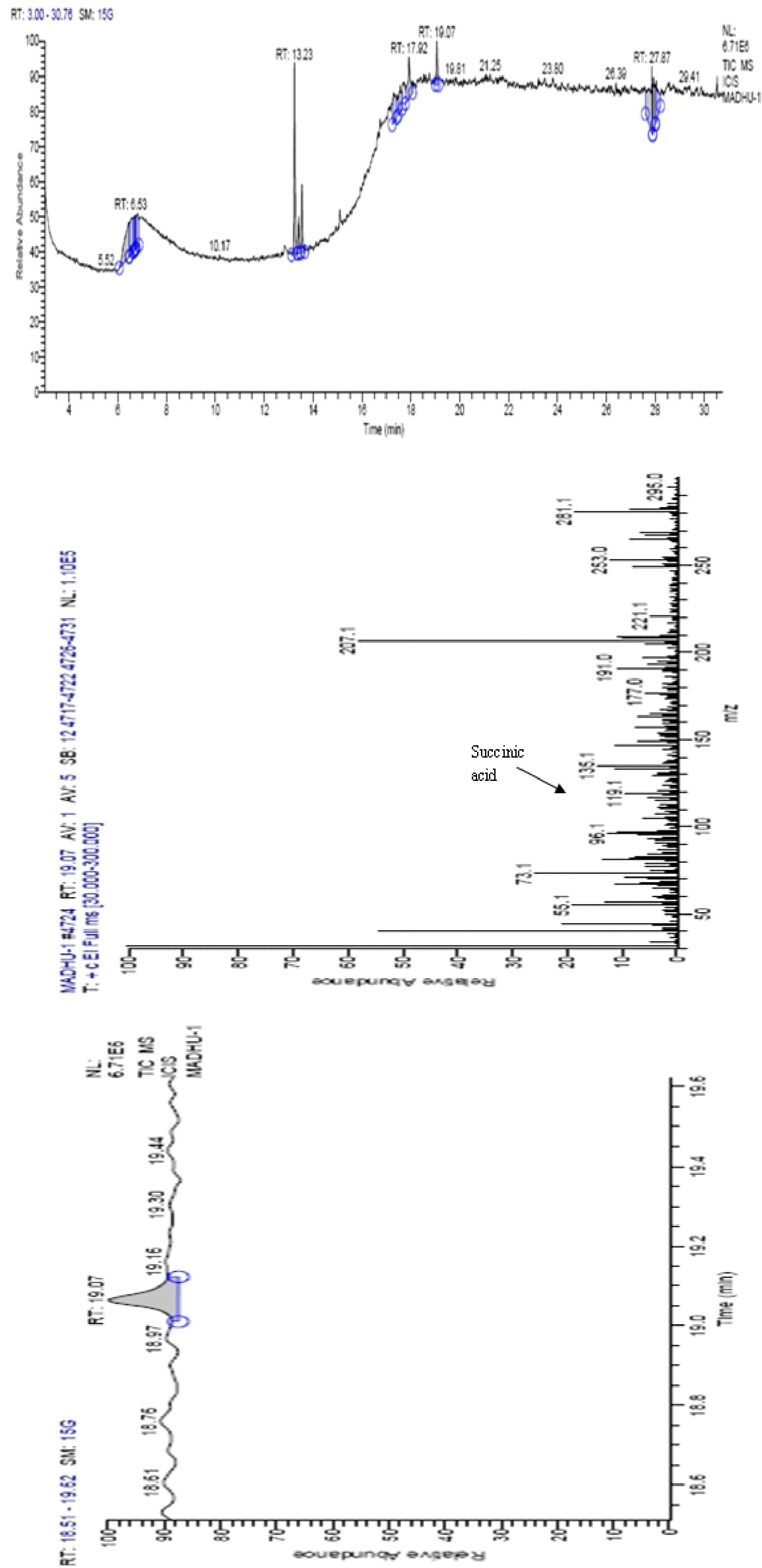

Fig 11 a: GC Profile of the biotransformation of Succinodinitrile into Succinic acid carried out using M. paraoxydans RS15 (b) Tandem MS profile of the peak at the retention time of 19.07 showing a peak of Succinic acid of (m/z) of 119.1

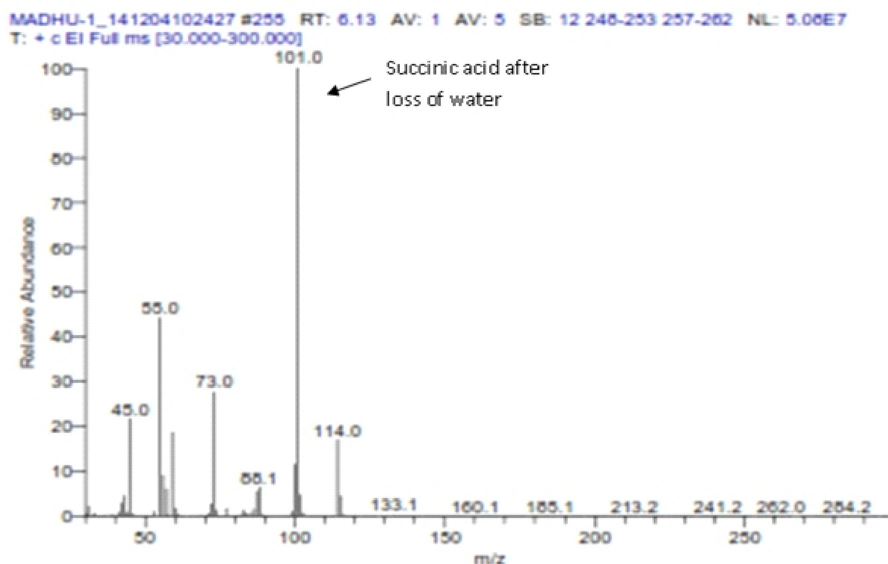

Fig 12: GC-MS graph of Succinic acid (pure compound) showing a peak of 101.1 (m/z) after the loss of a water molecule at 100% abundance.

substrate.

The nitrile hydrolysing enzyme production from RS15 in the shake flask experiment was quite enterprising. It increases with the addition of media supplements. But a few literatures are available regarding the mass production of nitrile hydrolysing enzyme. So, Scale up was done in 5L Bioreactor with and without media supplement.

A really comparable maxima in the activity was observed. It was 570 U/mg/ml after 11 hours (Fig. 5). The maximum absorbance was 0.174 at 11 hours which makes it to conclude that this culture was successfully scaled up. The fermentation condition were kept alike as shake flask experiment at 30°C at 180rpm. The aeration rate was 0.4 LPM. It may be due to good aeration and agitation the maximum activity and growth was achieved at 11 hour after inoculation.

Growth and nitrile hydrolyzing enzyme production of RS15 in *in-situ* bioreactor using MSM+ 25 mM glucose + 25mM succinodinitrile as substrate.

The maximum enzyme activity obtained after adding glucose at scale-up level was 262 U/mg/ml at 6 hours. The maximum absorbance of cells was 0.663 at 7 hours (Fig. 6).

Kinetic characterization

Temperature optimization

The temperature maxima of RS15 enzyme was 237U/mg/ml, at 30 °C (Fig. 7). Activity relative to 30°C was plotted against temperature in it. At 20°C and 50 °C, the relative activity was reduced to 0%, while nearly half optima was observed at 35 °C.

pH optimization

As temperature optima was at 30 °C, the pH optimization was also done at 30 °C. Different buffers phosphate buffer, Tris- HCl buffer of 200mM concentration were used for it for different pH 5-10. Fig. 8 showing the pH optima at 7. The enzyme was quite stable at slightly acidic pH while half- optima was achieved at pH 8. The activity was significantly decrease at pH 10.

Thermostability

After studying the temperature optima thermal stability was studied for 6 hours. For this crude intracellular enzyme was incubated at 30 °C and 40 °C upto 6 hours. Fig. 9 clearly indicated that enzyme is very much stable at 30 °C rather than 40 °C. Due to this half optima was achieved after 5 hours at 30 °C while it was achieved only after 2 hours at 40 °C.

Solvent stability

Fig.10 is showing the effect of solvents on the whole cell enzyme. The cells were exposed to four different solvents: Hexane, Petroleum benzine, toluene and Ethyl acetate. Hexane and petroleum benzine were more non- polar as compared to ethyl acetate. In hexane 64% of the original activity was retained by the enzyme during first two hours, followed by further decrease in activity. In case of petroleum benzine 49% of the original activity was retained in 2 hours. In ethyl acetate the original activity was reduced to 55% in first 2 hours. In toluene 21% of original activity retained in first two hours. The actual or control activity was 319U/mg/ml.

GC-MS

The activity of *M. paraoxydans* RS15 to catalyze the hydrolysis of Succinodinitrile to the corresponding Succinic acid was further confirmed by characterization of the hydrolysis products. Biotransformation of Succinodinitrile by this bacterium was done. The products (especially Succinic acid) formed by the biotransformation reaction were analyzed by GC-MS, and identified by comparing the data with those of the standard samples. Fig. 11 (a) showed the GC profile of the products. In (Fig. 11 (b)) MS profile of the peak at a retention time of 19.07, there is Succinic acid with a mol wt. of 119.1 at an abundance of 15 %.

DISCUSSION

Microbial nitrilases have gathered immense attention because of an effective environment friendly alternate to convert nitrile waste into valuable and cheaper products with efficiency and

speed [12]. In search for the microbial nitrilases that have potential to do biotransformations, we had screened 40 nitrilase producing organisms on the basis of their growth on different compounds. After screening, the bright yellowish *M. paraoxydans* RS15 nitrilase was chosen to biotransform Succinodinitrile into Succinic acid. *M. paraoxydans* RS15 gave 507 U/ml/mg/min nitrilase activity. To enhance the activity of the enzyme various enhancers had been used by various researchers [13]. Glucose, glycerol, fructose, sodium acetate, starch, mannitol etc. had been used as carbon sources while yeast extract, phenylacetonitrile, peptone etc. has been used as the nitrogen source in the enrichment medium [14-19]. In this study, glucose and sodium citrate had been used as enhancers as well as carbon sources at shake flask level. But rise in the activity with sodium citrate was not as much as with the glucose. So, it means glucose acts as better media supplement than sodium citrate. Upscaling of the nitrilase producing microorganism *M. paraoxydans* RS15 was done in the 5L *in-situ* bioreactor from 250 ml flask level. The scale up of the nitrilase activity was done successfully as activity was enhanced to 570 U from 507 U. Similarly, Glucose was used as the carbon source at upscale level. The growth (absorbance) and maxima in the nitrilase activity were achieved early than without media supplementation with glucose. Besides the upscaling, stability of the enzyme also plays an important role in the commercial viability of the enzyme. Temperature, pH optima and solvent stability are the important kinetic parameters to study the enzyme. *M. paraoxydans* RS15 enzyme had 30 °C and pH 8 as its temperature and pH optima respectively. The activity decreased more rapidly with decrease in temperature rather than its increase. The nitrilase enzyme achieved half temperature optima after 5 h at 30 °C and was more stable at acidic pH (Fig. 8). Solvent stability of the enzyme was performed with four solvents i.e. hexane, petroleum benzine, toluene and ethyl acetate. Solvent stability profile of an enzyme helps to use the enzyme in non-aqueous enzymology [20]. Different solvents have different log P values, so have different effect on the enzyme pocket rigidity [21]. *M. paraoxydans* RS15 was more stable in hexane as compared to other solvents (Fig.10). Finally, the whole cell nitrilase of *M. paraoxydans* RS15 was used to biotransform Succinodinitrile into Succinic acid. The availability of Succinic acid was confirmed with Fig. 12 showing graph of Succinic acid after the loss of a water molecule at an abundance of 100%. The product with 207.1 retention time can be 4-Benzylphenylacetonitrile, which can also be used as an important compound.

CONCLUSION

A mesophilic nitrile hydrolysing strain, *Microbacterium paraoxydans* RS15 was isolated, which was able to grow upto 200mM concentration of Succinodinitrile. GC profile showed the biotransformation of Succinodinitrile to Succinic acid by the enzyme of this organism. It has been successfully upscaled to laboratory scale bioreactor. Its activity has been increased prominently by a cheaper source like Glucose, which promises to use this culture at industrial scale level at a low cost.

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