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Efficient Utilization of Dairy Industry Waste for Hyper-Production and Characterization of a Novel Cysteine Protease

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Abstract.- Dairy industry waste (fresh cheese whey) has been utilized as a sole substrate for the production of extracellular protease by a locally isolated strain of *Rhizopus oryzae.* In pure whey medium only mineral salts were added as (g/L): $MnSO_4.H_2O$ (0.01%), $FeSO_4.H_2O$ (0.01%), $CaCl_2$ (0.05%) at pH 6. Effects of shaking speed, pH, temperature and incubation time for protease production were studied in submerged fermentation process. Maximum production of extracellular alkaline protease was achieved after 168 h at 35°C. To further improve the production from this strain mutagenesis was performed. Strain improvement using EMS culminated in a mutant ROAC5 with a 2.2 fold increased yield and low volumetric substrate consumption. Partial characterization was performed to expose the enzyme properties, obtained from parent and mutant strains. ANOVA confirmed that the enzyme produced from mutant strain was novel, thermo-tolerant and active at pH 10, resulting in 3.85 fold elevated enzyme activity (2538.9 PU/ml), under optimum fermentation conditions. This is the first study to report a novel cysteine alkaline protease produced by *Rhizopus oryzae*.

Key words: Whey, *Rhizopus oryzae*, cysteine, chemical mutation, protease.

INTRODUCTION

Whey is a major by-product of dairy industry in Pakistan as well as worldwide. It is a milk serum obtained after coagulation of milk during cheese manufacturing or butter extraction process and usually contains 4-5% lactose, 0.8-1.0% proteins, some minerals and vitamins. Due to its high biochemical oxygen demand (BOD), whey disposal is a costly and time consuming process. Its direct disposal into sewerage water causes inherent danger of water pollution (Ashraf *et al*., 2008). Being a cheaper organic carbon and nitrogen source, whey utilization can be envisaged for production of an assortment of value added products like proteases for use in food, pharmaceutical, beverage and textile industry (Rao *et al*., 1998).

Proteases are one of the major groups of industrial enzymes occupying diversified usage in a number of industrial applications (Banerjee *et al.*, 1999). These are protein digesting enzymes belonging to class hydrolases. Physiological importance of protease can be delineated by their involvement in processes like enzyme modification (Tanimoto *et al.*, 1991), regulation of metabolism, gene expression, blood coagulation, pathogenicity and release of hormones within the body. Alkaline proteases are largely involved in industrial applications *e.g.*, textile finishing, detergent additives, leather dehairing and pharmaceutical industries. Apart from aforementioned applications, contact lens cleaning solutions, nucleic acid isolation, silk degumming and hemp delignification processes also make use of alkaline proteases (Gupta *et al.*, 2002; Sumantha *et al*., 2006).

Regarding the sources of production, proteases can be obtained from plants, animals and microorganisms. Current production of these functionally important industrial enzymes is usually through microbial sources because they offer an advantage for being economic substitute and conservation of natural populations of plants and animals (Gupta *et al.*, 2002; Devi *et al*., 2008). Bacterial alkaline proteases are usually discouraged

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due to requirement of cost intensive procedures for their separation (Devi *et al*., 2008; Joo *et al*., 2001). On the other hand, molds offer an advantage over bacteria due to their growth on natural substrates and mainly the simple filtration process for separation of mycelia as described by Devi *et al*. (2008) and Joo *et al*. (2002). *Rhizopus oryzae* is a known producer for an assortment of products such as lactic acid, fumaric acid, amylases and lipases (Ikasari and Mitchell, 1998; Sumantha *et al*., 2006). Apart from this, many authors have reported the production of acid, alkaline and neutral proteases by *Rhizopus* sp. (Kumar *et al*., 2005; Aikat and Bhattacharyya, 2000; Egusa and Otani, 2009) on agro industrial residues. In addition to enzyme production on wheat bran, rice bran (Sumantha *et al*., 2006) and other agro- residues, *Rhizopus oryzae* has the ability to utilize lactose as its sole carbon source.

Current study accounts for a detailed description of the process for the production of alkaline protease via locally soil isolated strain of *Rhizopus oryzae* and optimization of the production process by the deployment of low cost organic media in submerged fermentation process. Fresh clarified cheese whey was employed to keep the process economically feasible. Strain improvement and subsequent partial characterization of the alkaline protease was also investigated for its potential in industrial scale application.

MATERIALS AND METHODS

Fermentation medium

The fresh cheese whey was kindly provided by Idara-e-Kissan Halla Milk & Dairy products, Lahore, Pakistan and used throughout this study as fermentation medium. To remove the suspended particles contained in raw whey, filtration step was performed by Whatmann No. 1 filter paper. Supernatant obtained after filtration was termed as "clarified/pure whey".

Microorganism and inoculum preparation

A soil isolated strain of *Rhizopus oryzae* was used in this study. Isolated culture was cultivated and maintained on Potato Dextrose Agar (PDA) at 30°C and 4°C, respectively.

Inoculum was prepared by dispersing spores in sterile distilled water from three day old slant. Spores were detached from mycelium with sterilized inoculation loop and then transferred into autoclaved distilled water. Suspension was diluted to 10^7-10^8 spores/ml. This spore suspension (2%) was used to inoculate the fermentation medium for protease production.

Submerged fermentation

Pure whey with added salts was used as fermentation media with following composition (g/L); $MnSO_4.H_2O$ 0.1, $FeSO_4.H_2O$ 0.1 and $CaCl_2$ 0.5. Medium was sterilized at 121°C for 20 minutes. Extracellular protease production was done in shaking water bath at 30°C. Crude protease enzyme was separated from mycelium by Whatmann No. 1 filter paper for analytical assays. All the experiments were performed in triplicate.

Optimization of fermentation parameters

One-factor-at-a-time optimization strategy was used for characterization of fermentation parameters. Fermentation parameters optimized were shaking speed, time course, initial pH (pH_o) and temperature. All the experiments performed in triplicate for the analysis of enzyme activities.

Mutagenesis

UV mutation

Serial dilution of the spore suspension was prepared in distilled water to get 10^8/ml spore suspension. 1 ml of above spore suspension was poured in petri dish and subjected to UV irradiation for 0, 15, 30, 60 and 90 minutes at a 10cm distance. For stabilization of thymine-thymine (T-T) dimers plates were kept in the dark for minimum 30 minutes. 0.1% sodium deoxycholate supplemented PDA was poured in plates containing irradiated spores, mixed well for even distribution, solidified and incubated at 37°C. One putative mutant from each treatment was then selected by qualitative and quantitative assay of enzymatic activity.

Chemical mutation

One ml of spore suspension (10^8/ml) was centrifuged to get spore pellet. Spore pellet was suspended in 1 ml (4µl/ml) of ethyl methane sulfonate (EMS) and exposed for 0, 15, 30, 60, 90, 120 and 150 minutes. After chemical exposure spore were washed twice with sodium thiosulphate and once with distilled water, poured on plates, mixed well with liquid PDA media supplemented with 0.1 % sodium deoxycholate and incubated at 37°C after solidification. Activities of mutants from each treatment were compared and best mutant was chosen for further study.

Partial characterization of crude protease

Characterization of an enzyme is crucial to evaluate the specific parameters for its maximum activity. Crude enzyme was partially characterized to study the influence of different parameters on its activity. For this, pH (7, 8, 9, 10, 11), incubation time (10, 15, 20, 25, 30 min), temperature (31, 34, 37, 40, 43, 46 and 49°C) and effect of metal ions were studied.

Effect of metal ions on enzyme activity was studied by using variety of metal ions *viz.*, Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} and PMSF. 100 µM solution of each metal ion was prepared and preincubated with crude enzyme for 20 minutes. Residual enzyme activity was determined by the method described in next section.

Analytical procedures

To measure protease activity of the crude enzyme filtrate, modified Anson method was used (Yang and Huang, 1994). The reaction mixture containing 1 ml of crude enzyme extract and 2 ml of casein solution (1%) in Tris-HCL buffer (0.1 M, pH 8.0) was incubated at 37°C for 20 min. Reaction was arrested by the addition of 3 ml trichloroacetic acid (10%). Absorbance of the clear supernatant was recorded at 280nm using tyrosine as a standard. One unit of enzyme activity is defined as the amount of enzyme liberating 1µg tyrosine per ml under the assay conditions.

For total protein content estimation and total carbohydrate estimation methods cited elsewhere were employed (Lowry *et al.,* 1951; BeMiller, 2003).

Kinetic studies and statistical analysis

Kinetic parameters include product yield Yp/s, specific product yield Yp/x, volumetric productivity Qp and volumetric substrate uptake rate Qs by Lawford and Rousseau (1993). In statistical analysis, two-way ANOVA was used to measure statistical significance of each variable for enzymes produced from parental and mutant *Rhizopus oryzae* strains with Minitab 15.0 trial version.

RESULTS AND DISCUSSION

Optimization of fermentation parameters

Agitation speed

Result of the shaking speed show that at 120 rpm, highest yield in terms of specific productivity was attained. Maximum volumetric production of the enzyme occurred at the expense of minimum substrate consumption (Table I), owing to its contribution towards larger amounts of product per liter of substrate consumed. Agitation/shaking impart significant role in biomass growth and the product yield. Highest production was achieved at 120rpm (Table I). Different morphological forms of *Rhizopus oryzae,* from suspended mycelia to small pellet formation, were observed in this study on different shaking speeds (120-140rpm). Macroscopic morphology of the organism and hydrodynamic forces influence the formation of product according to Kelly *et al.* (2004) and Ryoo (1999). In addition, inoculum spore count, metal ion, pH, medium composition (Elmayergi *et al*., 1973) and finally the agitation speed in a fermentation process also contribute to the morphological form as well as extracellular product secretion by the filamentous fungi. Some strains such as *Rhizopus* sp. need strong agitation (190rpm) to form pellets (Zhou *et al.,* 2000; Metz and Kossen, 1977; Liao *et al.,* 2007). Presence of metal ions in fermentation medium may also exert on pellet formation was observed and it was as according to study by Liao *et al.* (2007).

Production time

Time course study revealed that biomass

growth reached optimum at 96 hr, remained stationary for two days and then followed a decline (Fig. 1). Unlike to biomass growth, protease yield (Yp/s) and volumetric productivity (Qp) peaked till 168 hr and then decreased (Table II), explaining a non-growth associated protease production. It points toward the phenomenon of autolysis, in which cell mass is degraded by the cellular enzymes and other cellular contents are released into the medium. This fact was illustrated by earlier studies and proved the non-growth associated production of protease where product maxima was achieved at end of fermentation time than cell mass maxima (Aikat and Bhattacharrya, 2000; Ustáriz *et al.*, 2008; Yang and Lin, 1998; Yang and Huang, 1994). Aikat and Bhattacharrya (2000) reported the maximal production of protease by *Rhizopus oryzae* after nine days while maximum fungal growth was at third day of fermentation.

Table I.- Comparison of parameters at different shaking.

Parameters	Shaking speed (RPM)		
	100	120	140
P (U/ml)	615	659.2	600.34
X (g/L)	9.5	10.01	9.3
$Y_{p/s}$, (U/mg)	1.046	1.125	0.856
$Y_{p/x}$, (U/g)	64.60	65.92	64.55
Qp (U/L/hr)	3664.3	3923.8	3571.1
Qs (g/L/hr)	3.5	3.48	4.172

Terms used: X, cell mass; P, proteolytic activity in U/ml; Yp/s, product yield (units of protease produced/mg of substrate consumed); Yp/x, specific product yield (units of protease produced/g of biomass); Qs, substrate consumed in g/L/h; Qp, protease produced /L/h.

Table II.- Effect of fermentation time on protease production.

Fermentation time (hr)	$Y_{p/s}$ (U/mg)	$Y_{p/x}$ (U/g)	Qp (U/L/h	Qs (g/L/h)
24	0.179	52.9	4562.1	25.54
48	0.198	21.57	3941.6	19.91
72	0.256	15.72	3722.2	14.56
96	0.354	19.88	3937.5	11.11
120	0.457	25.29	3898.16	8.94
144	0.535	31.45	4010.5	7.59
168	0.616	39.74	4021.4	6.529
192	0.5	62.2	2915.6	5.85

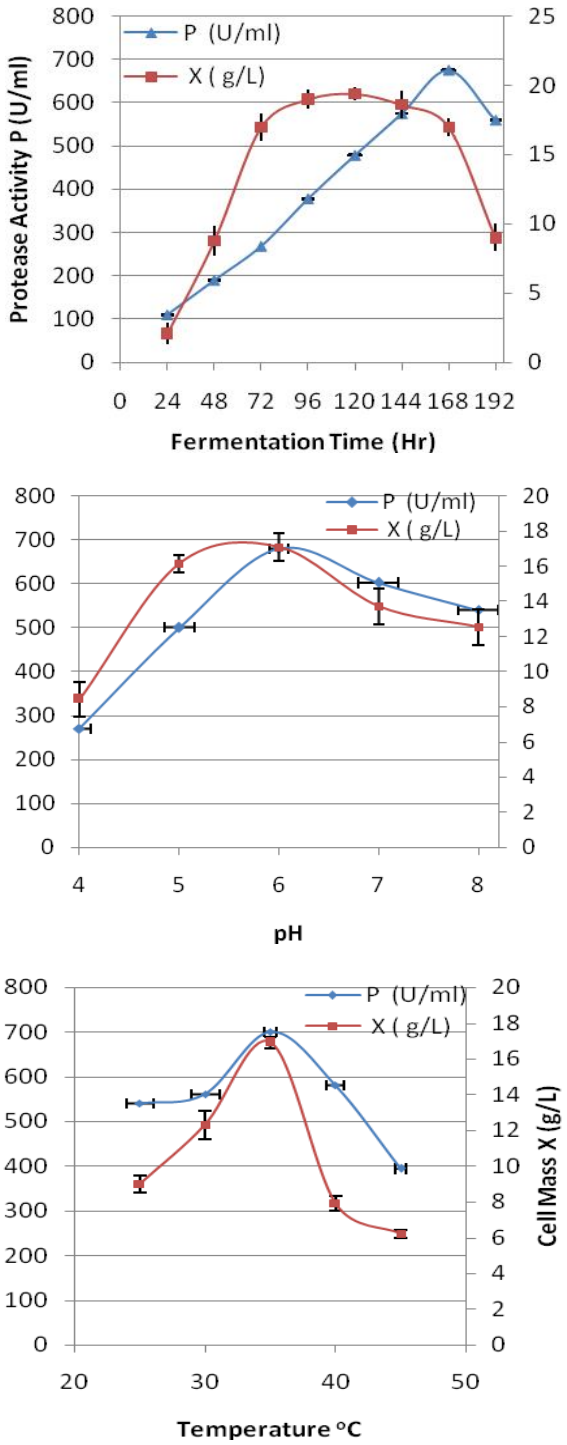

Fig. 1. Effect of time (hr), initial pH and

temperature of fermentation on biomass mass X (g/L) and protease activity P (U/ml). All the data are mean ± standard deviation, n=3.

Initial pH_o

Initial pH (pH_o) influence the growth and product yield in a variety of ways. A pH range 4-8 was employed to observe its effect on fermentation (Fig. 1). Maximum biomass and protease yield were obtained at pH 6.0. Change from this pH_o value affected the volumetric productivity (Qp) as well as volumetric substrate uptake rate (Qs) (Table III), although Liao *et al.* (2007) investigated that pH 3-7 do not affect the formation of *Rhizopus oryzae* pellets without emphasizing the type of product formed. The fact is that extracellular medium pH_o affects the intracellular enzymatic processes and the transport of nutrients across the cell membrane that takes part in cell growth and a specific product formation (Ellaiah *et al.*, 2002; Chi *et al.*, 2007).

Table III.- Effect of pH on protease production

Parameters	pH				
	4	5	6	7	8
$Y_{p/s}$ (U/mg)	0.268	0.52	0.67	0.61	0.545
$Y_{p/x}$ (U/g)	32.03	30.96	39.82	43.91	43.09
Qp (U/L/h)	1607.14	2976	4048.21	3583	3214
Qs (g/L/h)	5.99	5.73	6.04	5.84	5.99

Production temperature

Rhizopus oryzae being a mesophilic fungus is very sensitive to temperature changes. Elevated biomass growth was observed at 35°C (Fig. 1) with maximum yield coefficients (Yp/s, Yx/s) and volumetric productivity (Qp). Beyond optimum fermentation temperature there was reduction in both, biomass growth and product yield (Yp/s). Temperature is directly related to the integrity of cell membrane and the transport of nutrients across it (Heller and Höfer, 1975). Lower temperature slows the nutrient transport across cell membrane that might have resulted in lower substrate uptake rate (Qs) and product formation (Qp) (Table IV). Higher temperature is attributed for the cellular damage and increased maintenance energy requirement that resulted in more total protein (data not shown) acting as a thermal protectant but lower protease yield. Reduction in protease yield occurred as the cell growth was suppressed and hence more energy was needed to maintain the cell from environmental stresses (Haq and Ali, 2006). Temperature may also affect the metabolic pathway enzymes that denatures at higher temperature and protease production was halted. This phenomenon may be termed as thermal inactivation. Slight increase in production (700 PU/ml) was achieved by process optimization after 168h, 35°C, pH_o 6.0 and at 120 rpm. Increased in production was achieved by mutation of the parent strain.

Table IV.- Effect of temperature on protease production

Parameters	Temperature (°C)				
	25	30	35	40	45
$Y_{p/s}$ (U/mg)	0.558	0.558	0.674	0.583	0.4
$Y_{p/x}$ (U/g)	37.8	45.57	41.45	73.44	63.56
Qp (U/L/h)	3214.3	3336.31	4169.6	3453.6	2345.83
Qs (g/L/h)	5.76	5.98	6.184	5.925	5.86

Mutant selection

Single colony isolation in case of *Rhizopus oryzae* is a critical step as it rapidly spreads mycelia on agar plate and fills the entire plate in a short time. Single colonies obtained on sodium deoxycholate supplemented PDA plate were isolated and then screened for the hyperactive enzyme producing strain. Four isolates from UV treatment and six isolates from EMS were screened for selection of hyper-producing strain (data not shown). Putative mutant was selected on the basis of kinetic parameters (Yp/s, Yp/x, Qp and Qs) on previously optimized fermentation conditions.

Mutant $ROAU_2$ (30 minute UV exposure) and mutant $ROAC_5$ (120 min EMS exposure) resulted in 1.73 PU/mg and 1.88 PU/mg elevated protease yield (Yp/s) respectively than the parent strain (0.6 PU/mg). $ROAC_5$ showed a 6.3-fold enhanced specific product yield and regarded as the best mutant strain. Lowest volumetric substrate uptake, minimum biomass formation and highest volumetric productivity rate for $ROAC_5$ further authenticated it the hyper producing mutant strain of *Rhizopus oryzae* (Table V).

Partial characterization of protease

Partial characterization of the extracellular protease secreted by three strains; parent, $ROAU_2$ and $ROAC_5$ was performed on crude enzyme filtrate based on its casein hydrolyzing ability.

Table V.- Comparison of protease production by wild and mutant strains

Parameters	Strains		
	Wild	ROAU2	ROAC5
P (U/ml)	700.5	1359.2	1419.1
$Y_{p/s}$ (U/mg)	0.674	1.88	1.7276
$Y_{p/x}$ (U/g)	41.45	125.16	263.95
Qp (U/L/h)	4169.6	8090.5	8625.6
Qs (g/L/h)	6.184	4.30	4.86
P (U/ml)	700.5	1359.2	1419.1

pH

Crude enzyme filtrate from parent and mutant strains $ROAU_2$ and $ROAC_5$ were partially characterized and their activities were compared by ANOVA. Variance in protease activity was observed with respect to both pH and strain. Protease from parent strain was active at slightly alkaline pH (8.0) but $ROAU_2$ and $ROAC_5$ were active at pH 10. $P<0.05$ indicates significant difference among the three enzymes; *i.e.* mutation has resulted in change in enzyme characteristics as well as the response to pH. At pH 10 confidence interval 95 % showed that response of enzyme from $ROAC_5$ was high (1260.58 PU/ml) than parent/wild and mutant $ROAU_2$ (Fig. 4). Genckal and Tari (2006) carried out serine alkaline protease production which was also active at pH 10. A study by Banerjee and Bhattacharyya (1992) supports the production of alkaline protease from *Rhizopus oryzae*.

Incubation time

Influence of incubation time was significant at $\alpha=0.05$. All three proteases showed similar response pattern to incubation time. Highly significant result was obtained with 97.22% and 95.13% R-Sq and R-Sq (adjusted) values respectively. The optimum activity of mutant $ROAC_5$ (1261.1PU/ml) was after 20 minute incubation at 37°C and pH=10.0 (Fig. 3). Enzyme from other strains also exhibited maximum activity after 20 minute incubation time at their respective pH values described above.

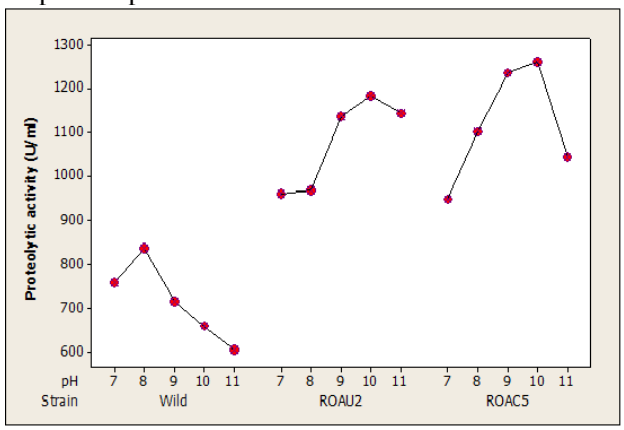

Fig. 2. Comparison analysis of effect of pH on wild, $ROAU_2$ and $ROAC_5$ protease. R^2=84.58%, R^2-adj=73.01% at 95% confidence interval.

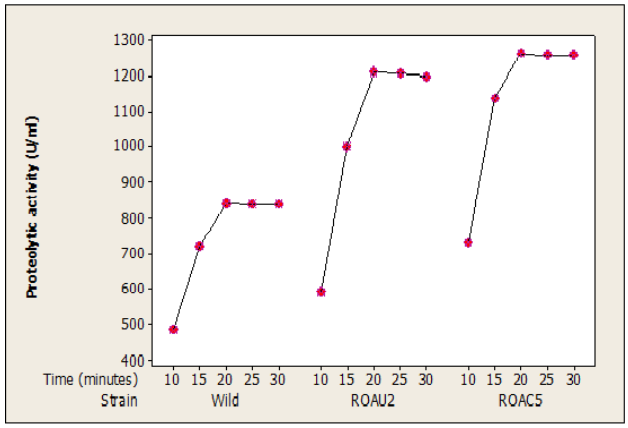

Fig. 3. Comparison analysis of effect of incubation time on wild, $ROAU_2$ and $ROAC_5$ protease. R^2 = 97.22% R^2-adj = 95.13% at 95% confidence interval.

Temperature

Maximum protease activity by $ROAC_5$ was observed at 46°C (Fig. 4). Although enzymes from all three strains were active above 40°C temperature but the activity of mutant $ROAC_5$ at 46°C was much greater than the parent at 40°C with R^2= 95.99% and R^2 (adjusted) = 93.18% demonstrating the presence of an extraordinary peaked proteolytic activity at

46°C. It points towards the secretion of a thermotolerant enzyme from mutant strain ($ROAC_5$) of *Rhizopus oryzae.* This result was consistent with the study by Olajyuigbe and Ajele (2005). Thermotolerant microbial enzymes are favorable for industrial applications. So this proteolytic enzyme can be used at industrial scale without substantial loss of activity at higher temperatures.

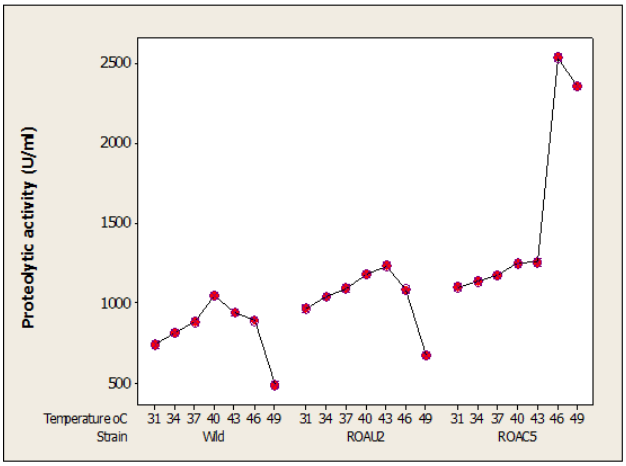

Fig 4. Comparison of effect of temperature on wild, $ROAU_2$ and $ROAC_5$ protease. R^2 = 95.99% R^2- (adj) = 93.18% at 95% confidence interval.

Effect of metal ions

The effect of metal ions showed that alkaline protease was not a metalloprotease. Generally alkaline proteases neither inhibited nor activated by metal ions/ reducing agents (Kim *et al.* 2001). Mg^{2+} and PMSF did not affect the residual activity of the protease, which indicates that it is not a serine protease. $ZnCl_2$ and $FeSO_4$ both severely affected the activity of enzymes and acted as strong enzyme inhibitors (Fig. 5). A neutral metalloprotease activated by Mn^{2+} and inhibited by $FeSO_4$ and $MgSO_4$ and chelating agents was described by Sumantha *et al.* (2006). Zn^{2+} acts as inhibitor of cysteine protease as reported in other studies (Damare *et al.*, 2006; Gaur and Wadhwa, 2008). From the results it can be concluded that this enzyme relates to class of cysteine proteases because it is substantially inhibited by Zn^{2+} and Fe^{2+} that are known metal ion inhibitors for this class of proteases (Gaur and Wadhwa, 2008). Serine and aspartic proteases have already been reported by *Rhizopus oryzae* (Ellaiah *et al.*, 2002; Tanimoto *et al.*, 1991).

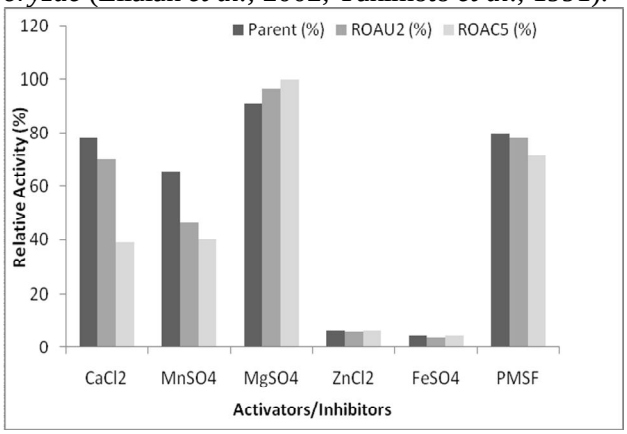

Fig. 5. Comparison of effect of metal ions on relative activity of protease from wild/parent, $ROAU_2$ and $ROAC_5$.

CONCLUSIONS

Industrial effluent spillage into sewerage water can be efficiently reduced by converting this hazardous by-product into an industrially important metabolite by microbial cell factories. *Rhizopus oryzae* exhibited enormous potential of consuming lactose as sole carbon source in whey besides fats and proteins. A locally soil isolated strain of *Rhizopus oryzae* proved itself an efficient producer with novel enzyme properties, greatly decreasing the BOD of whey in industrial effluent water. Whey medium devoid of any nitrogen/carbon supplementation proved to be an attractive low cost medium for protease production hence lactose serving as a sole carbon source. Product separation from fungal mycelium was easily managed through a simple filtration process, which accounts for another reduction towards product costs and its promising application in textile or leather industry. Moreover, as the whey is major contaminant of sewerage waters worldwide, this strain can be effectively utilized for bioremediation purposes. This is the first report about cysteine alkaline protease production by *Rhizopus oryzae* on industrial effluents. Further characterization and scale up studies are suggested to

exploit the full potential of the enzyme for other industrial applications.

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