

Scale Up of Production in a Bioreactor of a Halotolerant Protease from Moderately Halophilic *Bacillus* sp. Isolated from Soil

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ABSTRACT

Studies were conducted on the production of protease by moderately halophilic *Bacillus* sp. on agro-industrial waste materials. The bacterium could efficiently use many agro wastes as substrates but wheat bran supported maximum enzyme production. To ascertain the performance of the process in shake flasks and lab scale bioreactor, experiments were conducted to analyse protease activity utilizing wheat bran as cost effective substrate. The studies unveiled that pH 7.0, temperature 30 °C and static conditions were optimal for enzyme production in flask level fermentation. In scale-up fermentation, at optimal pH and temperature, agitation rate of 50 rpm was best for protease production. The enzymatic nature was studied in 10% SDS gels with BSA (2.5 mg/mL) as substrate and banding pattern was compared with undigested BSA as control. The endoprotease nature and the kinetics of protease activity were confirmed. The enzyme retained 37% of its activity even at 5 M NaCl concentration. The proteolytic activity was also confirmed by casein zymogram analysis. The fermentation medium containing inexpensive substrates, physical conditions and ability of *Bacillus* sp. to exhibit protease activity on a large scale could collectively be useful for commercial production.

Key words: Poultry soil, endoprotease, scale up fermentation, wheat bran, halotolerant

INTRODUCTION

Proteases from the microbial sources are preferred than from plant and animal sources, as they can meet the increasing demand and possess almost all the characteristics desired for various biotechnology industries (Gupta et al. 2002). Proteases from *Bacillus* species are the most important group of secondary metabolites that are commercially exploited in both physiological and commercial fields (Ferrari et al. 1993). Proteolytic enzymes find wide application in various industries such as food, detergent, unhairing and bating of hides, textile, wool quality improvement,

in tanning industry, pharmaceutical and leather processing (Prakasham et al. 2005; Saran et al. 2007). In nature, proteases have complex roles in executing the biochemical and physiological functions. Among bacteria, *Bacillus* strains are the most important producers of commercial proteases (Tegge 1985). They can be produced under extreme conditions such as temperature, pH, nutritional and salt concentration giving rise to value-added products that, in turn, are stable in a wide range of extreme environment (Han et al. 1997).

Different optimization techniques are used to determine the suitable operating parameters

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(Milavec et al. 2002; Xu et al. 2006). Thirty to forty percent of production cost of industrial enzymes is estimated to be the cost of growth medium (Jo et al. 2002). Therefore, it is important to optimize the substrate and conditions for cost efficient enzyme production.

Advances in industrial biotechnology offer potential opportunities for economic utilisation of agro-industrial residues. Wheat bran is one of the important by products of food industry. It can be efficiently utilized for the production of value-added products through fermentation biotechnology (Zulfiqar et al. 2009).

Halophilic proteases have wide applications in the processing of food, leather and detergents. As moderate halophiles are capable of growing under wide range of salt concentrations (optimal growth at 3-15%), they constitute a very interesting group of microorganisms with great potential use in biotechnology because of the activity at high salt concentrations (Ventosa et al. 1998). The high-salt tolerance of halophiles enables their cultivation under non-sterile conditions and thus leads to cost reduction (Margesin et al. 2001).

Industrial fermentations are moving away from traditional and largely empirical operations towards knowledge based and better controlled processes. The metabolic performance of a microbial culture in the bioreactor strongly depends on complex interactions of the various operating conditions. The production of enzymes in large-scale is mostly carried out in batch fermentation in stirred tank bioreactors. Both physical and biological factors are quite different in a fermentor compared to that in a shake flask. The selection of design conditions and operational procedures are very relevant in scaling-up process to ensure that the effect of variables on the process is the same. Up-scaling the fermentation processes from lab-scale to commercial units is challenging due to the difficulty in assessing the factors affecting the scale-up process during the cultivation. It is well known that microorganisms are more susceptible in large-scale environmental variables, such as pH, temperature, dissolved oxygen (DO) and composition of raw materials. These variables have an ample effect on the microbial cell growth and metabolite production (Yuh-Lih et al. 2002). It is well known that extracellular protease production by microorganisms in bioreactors is greatly affected by media components, physical factors such as aeration, agitation, dissolved oxygen, temperature;

inoculum density and incubation time (Gupta et al. 2002).

In view of the above, the present study was envisaged with following objectives:

1. Production of halo tolerant protease from *Bacillus* sp in inexpensive optimized media in shake flask
2. Scaling-up the process to be made commercially viable, and
3. Determining the nature of enzyme in the gel.

MATERIALS AND METHODS

Materials

Agar and other medium components were procured from Hi-media (Mumbai, India). All other chemicals used were of analytical grade and were purchased from either Merck (Mumbai, India) or SD Fine Chemicals (Mumbai, India). UV 160A (Shimadzu, Kyoto, Japan) spectrophotometer was used for the enzyme assay. The data were analyzed using Statistical Package for the Social Sciences (SPSS) 19 (SPSS Inc., Chicago, IL, USA).

Isolation, screening and identification of bacterium

The soil samples collected from the fish market, poultry and butcheries were diluted in sterile saline solution and plated on casein (0.5%) and peptone (0.5%) medium.

The plates incubated at 37°C were observed after 24 h for zone of hydrolysis which gave an indication of protease producing organisms. Gelatin plates were also employed for screening of protease producers (VijayAnand et al. 2002). Depending upon the zone of clearance, two strains were selected and identified by MTCC. One organism was identified as *Bacillus* sp. *KBRP-2* belonging to *B. cereus* cluster, which was routinely sub-cultured and maintained on nutrient agar slants.

Fermentation conditions

The enzyme production was carried out in optimized medium containing casein 1%, yeast extract 0.5% and NaCl 2.5%. Seed inoculum (1% and one day old) was added to each Erlenmeyer flask (250 mL) containing medium and the flasks were incubated at 30°C under static conditions. The cell-free supernatant obtained by centrifugation (10,000 rpm, 10 min) was used for determining the extracellular protease activity. The

growth kinetics was determined by optical density measurement at 600 nm along with protease production by *Bacillus* sp. in the medium containing 1% casein, inoculated with 1% overnight grown culture (initial pH of the medium 7.0), agitation speed 50 rpm, and 32°C in batch fermentation. Samples were drawn at every six hours interval up to 10 days (Nilegaonkar et al. 2007).

Protease assay

The protease activity was assayed according to Kunitz (1947) with some modifications. Briefly, enzyme solution was added to substrate solution (0.7% casein in 50 mM Tris-HCl buffer, pH 7.0) and the mixture was incubated at 50°C for 10 minutes.

The reaction was stopped by adding 2.0 ml of TCA mixture (0.11 M trichloroacetic acid, 0.22 M sodium acetate, 0.33 M acetic acid) and was kept at room temperature for 20 minutes. The content was centrifuged and the absorbance of the filtrate was recorded spectrophotometrically at 280 nm. Enzyme units were measured using tyrosine (0 - 100 µg) as standard. One unit of protease activity (U) was defined as the amount of enzyme, which released 1 µM of tyrosine per minute under the assay conditions.

Effects of different agrowaste based substrates on protease production

To find out the suitability of agro based waste as substrates for protease production, a variety of agro waste based substrates such as green gram husk, ground nut cake, sesame cake, wheat bran, rice bran and coconut cake were purchased from the local market and used as the substrate replacing casein in the growth media under submerged condition to study their effect on the protease production at basal media conditions including 1% inoculum level (Prakasham et al. 2005). The enzyme activity was measured at 24 h interval for protease production.

Scale-up studies in fermentor

Production was scaled up in BIOSTAT B-Lite sartorius fermentor with 1-L working volumes. The bioreactor was equipped with monitors to measure and control the variables such as foam, temperature, pH, stirring rate and dissolved oxygen. The vessel of the bioreactor was equipped with blade turbines. Olive oil was added as an antifoam agent.

Fermentation was maintained between 32 - 33°C at pH 7.0 ± 0.05. The present study investigated the influence of various parameters such as pH, agitation etc. on enzyme production in order to optimize the conditions for the production by the bacterium.

Characterization of crude protease

Crude enzyme was incubated in different molar concentrations of NaCl (5 M - 0.5 M) at 37°C for 1 h and assayed for protease activity.

The pH stability was determined by pre-incubating the crude enzyme extracts in different buffers (pH 5.0 - 11.0) at 37°C for 24 h. The pH was adjusted using the following buffers (50 mM): citrate phosphate (pH 5.0 - 6.0), Tris-HCl (7.0 - 9.0) Glycine NaOH (10.0 -11.0) and the activity was measured as in the standard assay above.

In order to determine, the proteolytic nature of the enzyme (endopeptidase or exopeptidase), 10 µL of BSA (Stock concentration 2.5 mg/mL) was incubated with 30 µL of lyophilized crude enzyme and was run on 10% SDS - PAGE with BSA as control (Jaysankar et al. 2010).

For determining the kinetics of enzyme action, 30 µL of crude enzyme was incubated with the substrate for 2, 5, 8, 10, 15 and 20 min; enzyme action was terminated by heating at 100°C for 5 min followed by 10% SDS-PAGE to analyze the banding pattern.

Casein zymogram analysis was done to confirm the proteolytic activity. The gel was casted with 1% casein and the samples were mixed with Laemmli sample buffer without reducing agent and loaded onto the gel.

The samples were electrophoresed and renatured in buffer containing 2.5% Triton X-100 in 50 mM Tris HCl and developed in buffer with 200 mM NaCl, 5 mM CaCl₂ overnight.

The gel was stained with Coomassie Brilliant Blue (CBB) R-250, destained with methanol: acetic acid: water (30:10:60) and examined for clear zone on a blue background indicating protease activity (Troberg and Nagase 2003)

RESULTS AND DISCUSSION

Detection of Proteolytic activity on plates

The isolates exhibited zone of clearance on casein peptone agar plates and each bacterium was examined for maximum zones, which were selected for further confirmations as shown in

Figure 1A. The gelatin plates after incubation were developed with mercuric chloride (15% w/v) in HCl (20%). A clear transparent zone indicated the hydrolysis of gelatin by extracellular protease, whereas the rest of the plate became opaque because of the coagulation of gelatin by mercuric chloride (Fig. 1B).

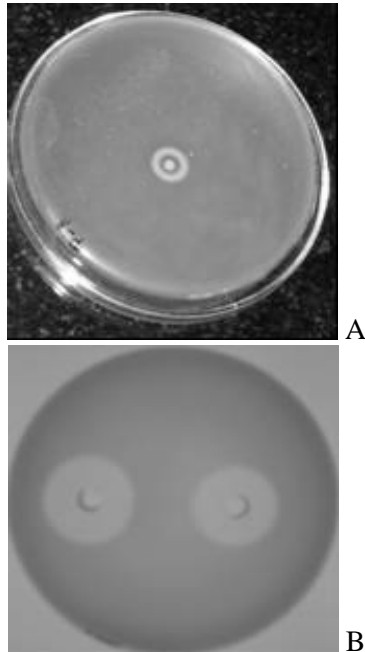


Figure 1 – 1A and 1B Zone of clearance on casein peptone and gelatin medium.

Cell growth and protease production

Bacillus sp. showed limited growth in 6 h and then entered the exponential phase. The synthesis and secretion started in the logarithmic phase after a day of inoculation and the stationary phase started after 54h. After the depletion of a critical growth substance from the medium, the increase in cell mass eventually ceased and the cells entered the stationary phase. Enzyme production was coherent with the growth pattern and it increased with the increasing growth. Substantial protease production started in this phase, the cellular metabolism changed and many products were synthesized which were of commercial interest as shown in Figure 2.

The lysis of cells in the late phase might have favoured the release of the enzyme. Similar observations were made and discussed by Singh et al. (2004) for *Bacillus sphaericus* secreting maximum proteases activity during post-exponential growth phase. A dehairing protease from *B. cereus*, reached to $126.87 \pm 1.32 \text{ Uml}^{-1}$ in 36h of the fermentation when the cell growth

reached late log phase or early stationary phase (Nilegaonkar et al. 2007). These differences in growth rate between the previous reports and the present findings might be due to differences in cultivation conditions as well as the *Bacillus* species.

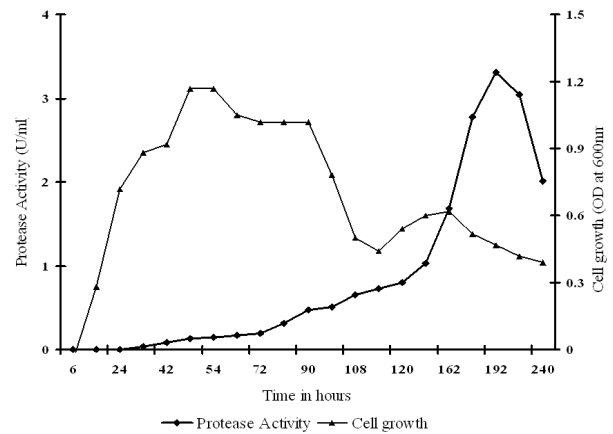


Figure 2 - Profile of cell concentration and protease activity of *Bacillus* sp.

Influence of different agro wastes as substrates

The selection of ideal agro by products for enzyme production in a fermentation process depends upon several factors, such as cost and availability of the substrate material, and thus it is highly relevant to screen for several agro-industrial residues. The effect of agro-based by products as alternative substrate on bacterial protease production under submerged fermentation was studied by several workers (Kaur et al. 1998). In the present study, casein was replaced by agro wastes such as coconut cake, sesame cake, groundnut cake and green gram husk for the cost effective commercial production of protease. The results indicated that the protease production varied with the type of agro waste. Wheat bran was the best inducer for protease production with maximum protease activity observed on the 7th day of incubation ($4.32 \pm 0.15 \text{ U/mL}$) with F value 85.6 and was significantly higher ($P < 0.01$) than observed with the other inducers of protease production as shown in Figure 3. In a similar study, reports describe use of agro-industrial residues for the production of alkaline protease, e.g. nug meal and *Bacillus* sp. AR009 (Gessesse 1997), pigeon pea and *Bacillus* sp. JB-99 (Johnvesly et al. 2002), wheat bran and *Rhizopus oryzae* (Aikat and Bhattacharyya 2000). Hence the effect of wheat bran concentration (1 - 5%) on the enzyme production was studied. It was observed that maximum activity $4.323 \pm 0.14 \text{ U/mL}$ of F value 27.85, in presence of 1% wheat

bran with was significantly higher ($P < 0.01$) and further increase in its concentration did not increase the activity (Fig. 4). In their studies on xylanase production by *Aspergillus niger*, Zulfiqar et al. (2009) found that wheat bran at concentration of 2.5% gave highest activity.

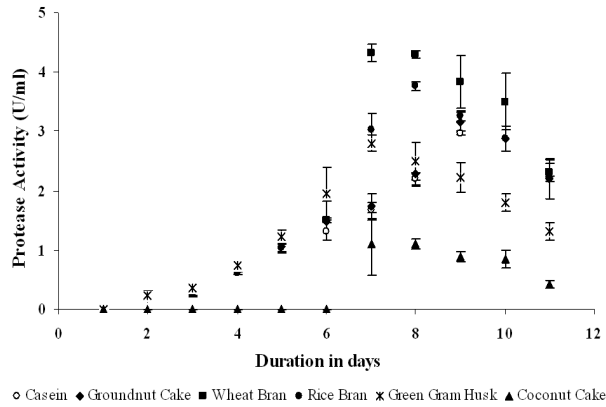


Figure 3 - Effect of different agrowastes on protease activity.

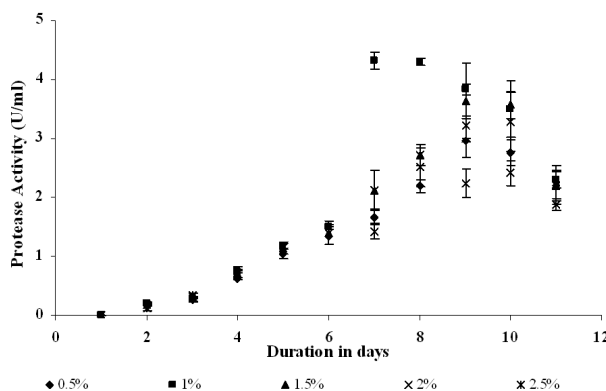


Figure 4 - Effect of different concentrations of wheat bran on protease activity.

Effect of pH and agitation rates in scale up fermentation

Effect of initial pH on protease production was investigated. The first batch of fermentation was performed under uncontrolled pH, the protease activity and pH was monitored every 24h. It was observed that on the first day there was a drop in the pH from 7.2 to 5.5 and thereafter the pH of the fermentation medium was almost constant till the end of fermentation. Maximum enzyme production of 2.85 ± 0.29 U/mL was obtained on the 8th day of incubation (Fig. 5). These results were beneficial for further studies, as it cut down the necessity to adjust the pH till the end of fermentation.

It is important to produce the enzyme in inexpensive and optimized media on large-scale for the process to be made commercially viable and also to study the influence of various parameters on enzyme production (SeetaLaxman et al. 2005). The production and timing of protease activity in the fermentations were investigated with respect to optimum agitation rate. Several fermentation batches were run at static, 50 and 100 rpm to optimize the agitation rate with pH, temperature and dissolved oxygen as constant parameters. Figure 6 showed that low agitation rate of 50 rpm (3.56 ± 0.07 U/mL) gave the maximum protease activity. Reduction in the activity at 100 rpm could be due to combined effect of stirring and aeration on enzyme inactivation.

For optimal enzyme production it is also necessary to reach a good mix of the culture broth since constant agitation produces a dispersion of air in the culture medium, homogenizes the temperature and the pH and improves transference rate of nutrients.

It has been reported that enzymes are susceptible to mechanical force, which may disturb the elaborate shape of complex molecules to such an extent leading to the denaturation of the molecules (Gupta et al. 2002). However, high speeds of agitation were not in favour of the enzymatic activity, which could probably be due to the shear stress caused by the blade tips of the impeller, which increases as the revolution speed increases (Pandey et al. 2000). Stress condition might contribute negatively toward cell growth and enzyme stability (Thomas et al. 1990).

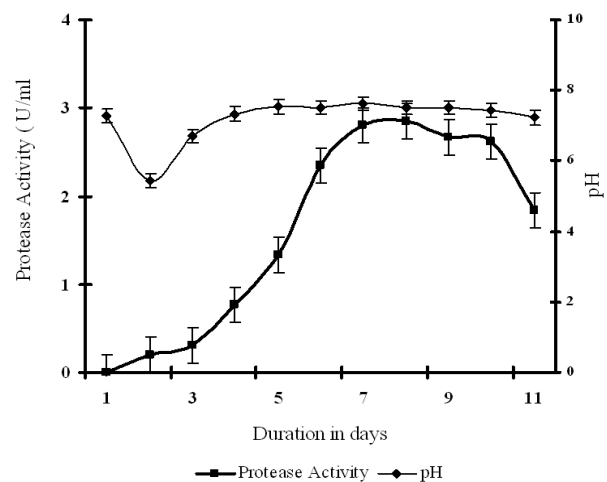


Figure 5 - Protease production in uncontrolled fermentation.

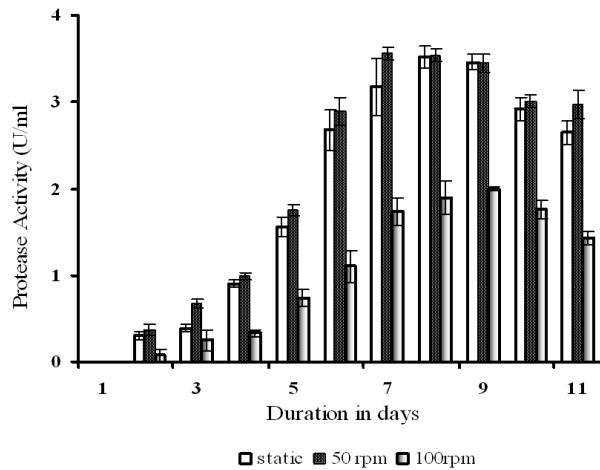


Figure 6 - Effect of agitation rate on protease production.

Characterization of crude protease

Crude enzyme was incubated in different molar concentrations of NaCl and assayed for protease activity. The enzyme retained 37% activity even at 5 M NaCl concentration demonstrating the nature of protease as halotolerant as shown in Figure 7. A number of moderately halophilic proteases can retain its activity even at higher salt concentrations.

They have substantial biotechnological applications particularly in the fermentation of foods. The pH profile of crude enzyme shown in Figure 8 revealed that protease activity was best in the pH range of 6.0 to 9.0, with maximum activity at pH 7.0 and no activity at pH 5.0 and 10.0 indicating that it to be a neutral protease and unstable at extreme pH. The neutral protease gene (npr) from *B. caldolyticus* was cloned and expressed in *B. subtilis*; the gene was found to be associated with the thermostability of the enzyme (Burg et al. 1991).

The BSA banding pattern on 10% SDS - PAGE revealed that the enzyme was endopeptidase. From the kinetics study, it was clear that BSA was completely digested within 2 minutes (Fig. 9A). Figure 9B shows the activity of protease on zymogram gel.

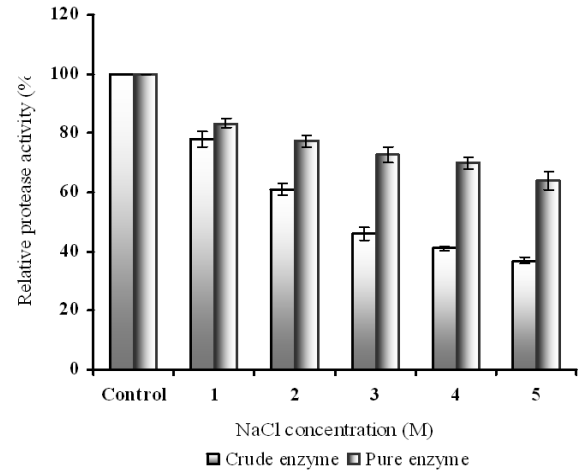


Figure 7 - Effect of NaCl on protease activity.

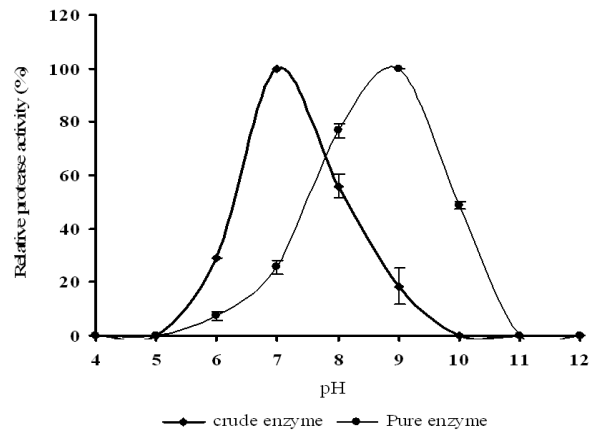


Figure 8 - Effect of pH on protease activity.

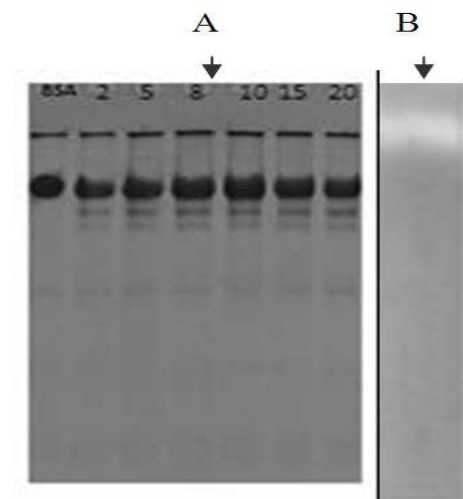


Figure 9 - A) BSA digested bands on 10% SDS PAGE. B) Hydrolysis zone upon 12% casein zymogram analysis.

CONCLUSIONS

It is important to produce the enzyme on large-scale in optimized and inexpensive media for the process to be commercially viable. The present study on the optimization and scale-up of production was an attempt in this direction. The results in the present study indicated that protease production pattern varied with the type of agro-waste and many agro wastes could be used by the organism as nutrient source for enzyme production. The enzyme production was scaled-up to 1.5 L in fermentor using wheat bran. The agitation rate as a variable for protease production was optimized. The agitation speed variation influenced the extent of mixing in the shake flasks or in the bioreactor and also affected the nutrient availability. Variation from these conditions resulted in lower yields of protease. The activity of crude protease on various concentrations of NaCl and at different pH was determined. *Bacillus* sp. (as the producer of protease) showed the potential for use in synthesis of bioactive peptides. Further studies on the purification of enzyme and its characterization need to be made for determining its use in bioactive peptide synthesis.

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Received: May 15, 2013;
Accepted: August 12, 2013.