Original Article

Efficient bioethanol production from paper shredder scrap by a marine derived saccharomyces cerevisiae C-19

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Abstract

The authors paid attention to paper shredder scrap as biomass and studied bioethanol production from them using marine derived yeasts. We reviewed current production methods, suggested the production method that minimized chemical agent use and machinery, and we found out additionally superiority of paper shredder scrap as a material. As for the saccharification of the material, it was compared sulfuric acid hydrolysis followed by enzymatic saccharification with only the enzymatic one. As a result, the amount of glucose production with the enzymatic saccharification was higher than that with the sulfuric acid followed by the enzymatic one. We found that glucose of 75 g was produced by only the enzymatic saccharification from the paper of 100 g. In this study, the saccharification solution of glucose concentration 270-290 g/L was used by all fermentation tests. About ethanol fermentation, a *Saccharomyces cerevisiae* C-19 derived from Tokyo Bay was mainly used and both a *S. cerevisiae* NBRC 10217 and a *S. cerevisiae* K-7 were used as control strains. As for the fermentation methods, the static fermentation, the agitating fermentation, and the repeated fermentations with immobilized yeasts using the alginate beads were performed. Furthermore, as a new way to increase the total amount of ethanol, ETS (Ethanol Trap System) was developed. By the agitating fermentation, the maximum ethanol concentration of 12 % (w/v) was observed using the strain C-19. In addition, 8.5 % (w/v) of ethanol concentration was produced by the repeated fermentation using immobilized yeast cells. In this study, we discussed both an optimum saccharification condition with enzymes and an optimum fermentation condition for ethanol production using the marine yeast strain C-19. Moreover, these results established that the paper shredder scrap was useful as a novel material for bioethanol production.

Key words

alginate beads, bioethanol, fermentation, paper shredder scrap, yeast

1. Introduction

In recent years, there has been a rapidly growing interest in bioethanol on a worldwide scale. Since development of an ethanol fuel becomes an alternative one to gasoline, it is expected to reduce greenhouse gas emissions, contribute to the prevention of global warming greatly. However, for instance, a corn was used as a main raw material in 2008 in America and grain prices started to rise globally. Additionally, a drought will make the American corn a bad harvest in 2012 and its use for bioethanol seems to be toublesome. That is, using agricultural products for ethanol materials would be accompanied by a risk.

Many researchers developed a cheap ethanol production method all over the world (Yan et al., 2006). Bioethanol studies using unused resources such as waste wood and rice straw have been accomplished in Japan. However, because lignin and pectin derived from plants are not suitable for bioethanol production, pretreatment of raw materials for their removal requires expensive equipment (Yang et al., 2008, and Wooley et al., 1999). To collect the plants from a vast mountain and fields, a huge cost is needed (Bharadwaj et al., 2007). Then, we considered using waste paper as bioethanol materials and paid attention to paper shredder scrap from them. Lignin and pectin are removed from the paper and starch is added in the paper manufacturing process. The cellulose which occupies most paper ingredients can be easily decomposed to small molecular substances such as glucose with enzymes. In addition, the general wastepaper can be reused, but the fiber of the paper shredder scrap is too small to be reconstructed. However, as the shredder paper is often dropped in urban areas, it is greatly worthy to use it, effectively without discarding.

In order to contribute to the effective use of industrial waste, we thought that the research about use of the shredder paper for a bioethanol material would improve environmental issues. In the past, bioethanol production using waste newspaper and paper sludge have been studied (Wang et al., 2012; Dubey et al., 2012; Insu et al., 2010). However, the ethanol production using paper shredder scrap have not been reported, yet. Because they are already coarsely crushed, there is a merit that pretreatment of the materials is easy.

Minato Ward around our university is a famous business district in Tokyo, Japan. The documents in corporate activity are usually put through a shredder not to leak information to others and large amounts of the paper shredder scrap are being discarded only in the district every day. The paper shred-

der scrap as raw materials for ethanol production have been overlooked until now. However, use of the materials would be the model for ethanol production because of their cellulose-rich construction. Therefore we looked for yeasts with high fermentative activities in the condensed saccharified solution and got a strain C-19 from Tokyo Bay. The marine yeast C-19 having osmotic pressure tolerance and ethanol tolerance could ferment the condensed saccharification solution of the papers, and the high efficiency ethanol production was possible.

2. Materials and Methods

2.1 Materials

The paper shredder scraps wasted as garbage in the university were used as materials. These papers were primarily collected from each laboratory. Most of the materials were the copy paper on which research content were written down. The paycheck stub, the thick paper such as the advertisement of the maker were mixed among them. Moreover, a lot of paper printed with the color ink was mixed, too. The kinds of the paper could not be distinguished, but the cardboard and the newspaper were not mixed.

2.2 Pretreatment for saccharification

The paper shredder scrap was roughly broken up. The paper was preliminarily saturated in the appropriate amount of DW (Distilled Water) at room temperature for 24 h and a paper fiber was expanded in order to crush itself easily. The DW 2000 mL and the paper shredder scrap 100 g were put into a food processor, then were broken up to moderately size (about 4 mm × 40 mm). Since a lignin and a pectin of the plant from wood pulp fiber had already been removed in the paper in the factory, neither heating nor medicine treatment for grinding is needed and only a rough crush was enough for cellulose expansion by absorbing water,.

2.3 Saccharification

2.3.1 Sulfuric acid hydrolysis followed by enzymatic saccharification

Dilute sulfuric acid (3 %v/v) 1500 mL and paper shredder scrap 100 g were put into a 2 L beaker, then high temperature high-pressure processing was carried out at 121 ℃ and for 1 h (Grohmann et al.,1986). Then the suspension was adjusted to 2 L with appropriate amount of water. After that, the hydrolysed suspension was neutralized to pH4.6 with NaOH solution, both 90,000 U of cellulase (Genencor International, Palo Alto, CA) and 49,000 U of *α*-amylase (Amano Enzyme, Japan) were added in the suspension, the saccharification was carried out for 2 days under agitation at 50 ℃,150 rpm. The amount of produced D-glucose was measured by an enzymatic method (F-kit, Roche, Basel, Switzerland).

2.3.2 Only enzymatic saccharification

One hundred g of the papers finishing pretreatment were put to a 2 L beaker, both cellulase (90,000 U) and amylase (49,000 U) were added into the suspension, the saccharification was carried out under agitation at 50 ℃ and 150 rpm for 2 days. The amount of produced D-glucose was measured by the same method as above. The saccharified solution was prepared by centrifuging at 4 ℃ and 3,000 rpm for 10 min to remove the residue from the suspension.

2.4 Condensation

The condensation of the saccharified solution was carried out to raise a glucose concentration by a rotary vacuum evaporator (EYERA N-N-P, Japan). The saccharified solution was put in a 2 L eggplant-type flask, kept warming below 40 ℃ in a water bath, sucked out with a vacuum pump (ULVAC DTC-41 ULVAC KIKO, Inc, Japan), the condensation was carried out under reduced pressure.

2.5 Yeast culture

Yeasts used in this study were a *Saccharomyces cerevisiae* strain C-19, which was identified by 26S rDNA D1/D2 domain sequence (DNA Data Bank of Japan: accesion no. AB767255), isolated from Tokyo Bay, a *S. cerevisiae* strain K-7 which is frequently-used in the Japanese sake brewing and, a *S. cerevisiae* NBRC10217 which is commonly used as a type strain (Takagi et al., 2012). The strains were cultured on YPD agar plates at 30 ℃ and kept at 4 ℃. The compositions of YPD medium were yeast extract 10 g/L, peptone 20 g/L, and D-glucose 20 g/L. The yeasts used for ethanol fermentation were cultured to ensure good activities every month. The yeast cells were used by doing a prior culture followed by a main culture in the fermentation. Each yeast grown at 30℃ for 1day in the prior one was added to YPD liquid medium 100 mL and cultured in the main one at 30 ℃ for 2days with shaking 120 rpm (YAMATO Incubator Shaker IK400W, Japan) .

2.6 Fermentation

In the static fermentation, 10 ml of saccharified solution (glucose concentration: 290 g/L) adjusted to pH 7.0 and yeast cells 0.2 g were added in a test tube. The fermentation was carried out in the test tube using an anaerobic jar (MGC Co. Ltd. Japan) at 30 ℃ for 7 days.

In the agitation fermentation, yeast cells 2.0 g were added to a 500 mL anaerobic flask containing the saccharified solution 100 mL (glucose concentration: 290 g/L) adjusted to pH7.0 and the fermentation was run in the flask with agitation at 100 rpm and 30 ℃ for 7 days. Then ethanol concentrations were determined by the enzymatic method (F-kit, Roche).

2.7 ETS (ethanol trap system)

Figure 1 shows the fermentation device with ETS (ethanol

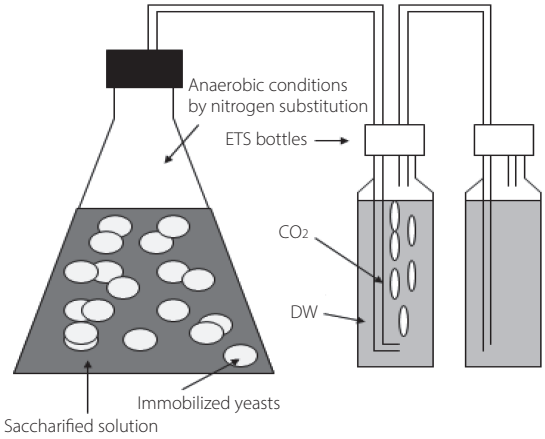

Figure 1: Pattern diagrams of ETS
CO_2 and vaporized ethanol was produced during fermentation. Vaporized ethanol was trapped in the first ETS bottle, and the ethanol was not detected in the second bottle. On the other hand, CO_2 moved to the second ETS bottle.

trap system). Two bottles for ETS containing 2 L DW were used to liquefy ethanol vaporized from the 500 mL fermentation flask. The bottles and the flask were connected with the silicon tubes which were extended to the bottom of bottles and were made for CO_2 bubbles to blow off. During the fermentation, CO_2 and vaporized ethanol passed through a silicon tube and the ethanol was dissolved into the water in the bottles.

2.8 Immobilization of yeast cells into alginate beads

Pellets of Yeast cells were obtained from the culture and immobilized into alginate beads. We used the original immobilization method using alginate by modification of the normal ones (Kwang et al., 2011; Svetlana et al., 2009; Masniroszaime et al., 2011). The solution containing $(NH_4)_2SO_4$: 2 g/L, $MgSO_4 \cdot 7H_2O$: 0.2 g/L , KH_2PO_4: 0.5 g/L , $CaCl_2 \cdot 2H_2O$: 0.01 g/L ,Yeast Extract: 5 g/L was prepared. Sodium alginate 3.75 g and the solution 150 mL were added to a 300 mL beaker, being heated with a micro wave oven, it was melted until it became translucent. And the yeast pellets 3 g was mixed with gelatinous alginic acid solution and cooled enough. The yeast strain used for immobilization was the C-19 or the K-7. Next, gelled liquid was injected into the syringe , and it was slowly dropped to a 1 % calcium chloride solution. Then, the immobilized yeast gel beads of 0.5 cm diameter were obtained.

2.9 Repeated fermentation by immobilized yeasts

The immobilized yeasts (wet 2.0 g) were mixed with 100 mL of the saccharified solution in a 500 mL flask. In order to make the inside of a flask into an anaerobic condition, a nitrogen substitution was done. The fermentation with ETS was carried out at 30 °C, 30 rpm for 10 days. The beads were washed by DW after one fermentation. Using the same beads, the fermentations were repeated 5 times with saccharification liquid of the same glucose concentration.

3. Results

3.1 Saccharification

After rough smashing of the paper shredder scrap, saccharification to convert cellulose into glucose is necessary. When cellulose is converted to soluble low molecular such as glucose, yeast can converted the substrate into ethanol. To compare 3 % sulfuric acid hydrolysis followed by enzymatic saccharification with only the enzymatic one, glucose productivities were examined. The former method was as follows: Put paper shredder scrap with 3 % dilute sulfuric acid, was hydrolyzed in the test tube at 121 °C and for 1 h, then the hydrolysate was saccharified with enzyme. The latter method was as follows: Paper shredder scrap was saccharified directly with enzyme instead of acid treatment. Paper ink was separated from paper shredder scrap in this way. The glucose 70.4 ± 1.9 g was generated from 100 g of paper shredder scrap by 3 % sulfuric acid hydrolysis followed by enzymatic saccharification. The glucose 75.2 ± 0.4 g was generated from 100 g of the paper shredder scrap by only enzymatic saccharification.

From the results, the high pressure and high temperate acid treatment are not needed for the saccharification of paper shredder scrap, we found that the papers was completely saccharified only by the enzymatic method. The results would lead to decrease the cost of bioethanol production from paper shredder scrap.

Table 1: Glucose production by two saccharification methods

Metnod	Amount of glucose (g)	Glucose yield
Sulfuric acid with enzyme	70.4 ± 1.9	70 %
Enzyme	75.2 ± 0.4	75 %

3.2 Condensation of saccharified solution

As the distillation process of fermented products requires a lot of energy, it is necessary to increase the glucose concentration and to reduce the amount of water as much as possible. The saccharified solution was condensed by rotary evaporator to raise glucose concentration. In this study we prepared a high condensed solution containing about 300 g/L of glucose from 200 g of paper shredder scrap. Glucose concentration can be increased up to about 500 g/L, but yeasts did not ferment it at the high level of glucose. Since rice straw and scrapped wood are contained high-viscosity materials such as lignin, it is difficult to condense their saccharified solution. We thought that the reason for the high level condensation in this study might be the removal of lignin and pectin at a paper mill stage.

3.3 Static fermentation

It was investigated how much ethanol was produced from a

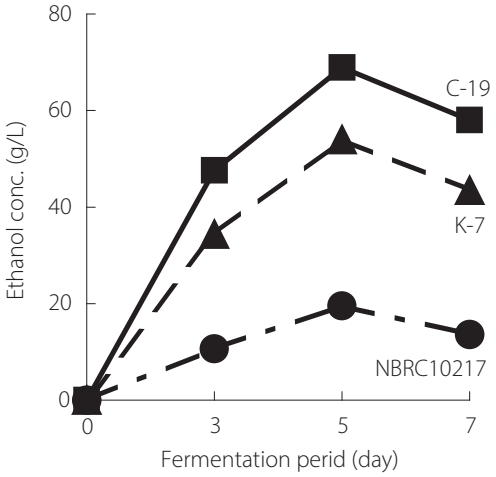

Figure 2: Ethanol production with each yeast strain in static fermentation

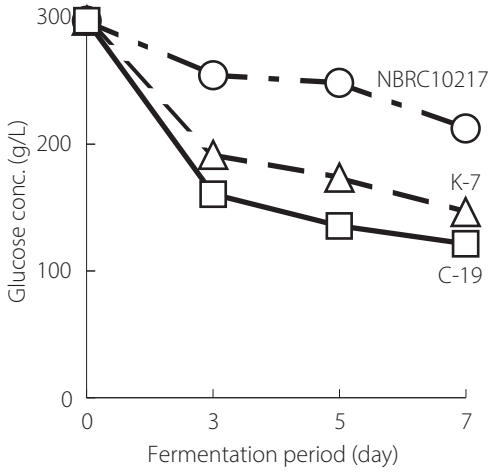

Figure 3: The amount of remaining glucose with each yeast strain in static fermentation

high condensed glucose solution from paper shredder scrap. The yeast *Saccharomyces cerevisiae* strain K-7, NBRC10217, or C-19 was used for the ethanol fermentation. The saccharified solution containing 297 g/L of glucose and yeast cells of 0.2 g were put into test tubes. Fermentation was performed for 7 days at 30 °C, pH7 in an anaerobic jar. Figure 2 shows that each strain had the highest ethanol productivity on 5th day of the fermentation and the maximum value was about 70 g/L with the strain C-19. Figure 3 shows that glucose in the products fermented with each strain decreased gradually with the progress of the fermentation. On the other hands, ethanol production with each strain increased during 0-5 days and decreased on 7th day of fermentation as shown in Figure 2. These phenomena seemed to be due to the self-assimilation of ethanol with yeasts or vaporization of ethanol from the fermented liquid.

3.4 Agitating fermentation

Agitating fermentation was carried out to test whether the efficiency of bioethanol production can further be increased. An advantage of agitating fermentation for ethanol production seemed to be the increase of frequency of contact among substrates and yeasts. The maximum ethanol concentration was 98.5, 122.5, or 37.5 g/L for the strain K-7, the C-19, or the NBRC10217, respectively, as shown in Figure 4. Above all, the time period, 3 days, reached the maximum ethanol concentration with the strain C-19 was quicker, compared with those, 7 days, with the other strains. From the results, we found out fermentation ability of the marine yeast strain C-19 was higher than those of the reference strains K-7 and NBRC10217. According to the increase of ethanol as shown in Figure 4, the remaining glucose in each solution decreased gradually with the progress of the fermentation (Figure 5).

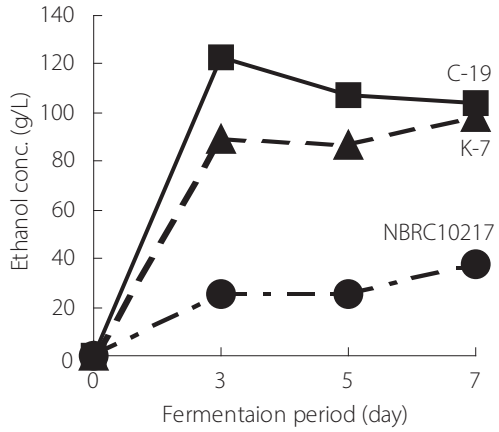

Figure 4: Ethanol production with each yeast strain in agitating fermentation

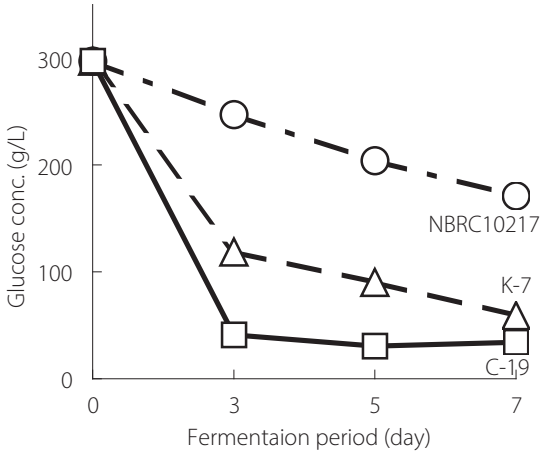

Figure 5: The amount of remaining glucose with each yeast strain in agitating fermentation

3.5 Repeated fermentation using immobilized yeasts

The repeated fermentation using immobilized yeasts was carried out in the condensed solution of glucose (270 g/L) as

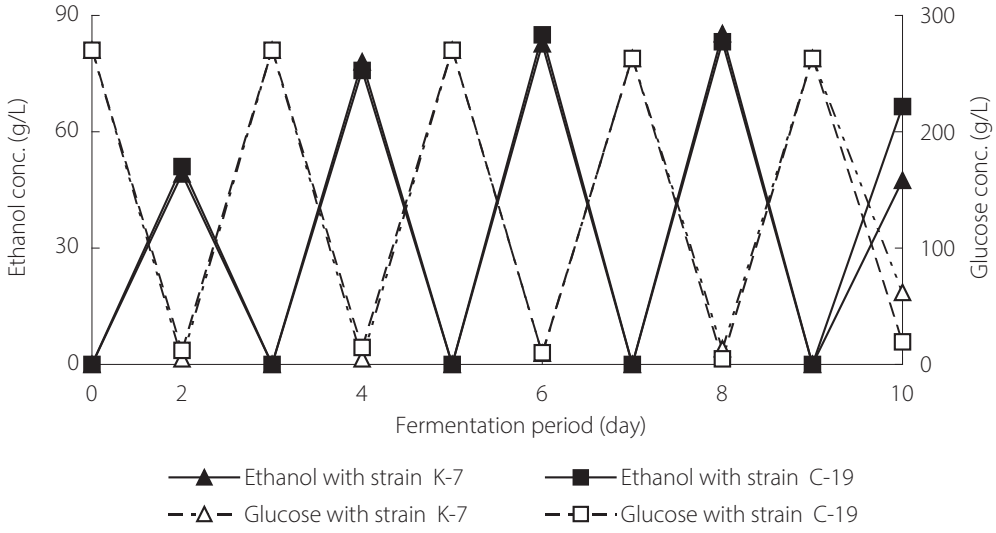

Figure 6: Repeated fermentation using immobilized yeast

shown in Figure 6. In the first-time fermentation, ethanol of about 50 g/L was produced with each immobilized strain. In the repeated fermentations of from 2nd to 4th times, ethanol of 80-85 g/L was produced with each one. In the last 5th, immobilized yeast strain C-19 or K-7 produced ethanol of 66.5 g/L or 47.5 g/L, respectively. On the other hand, a remaining glucose after every fermentation had decreased to about 1% or less. Consequently, the fermentation using yeasts immobilized into alginate beads was able to produce high concentration of ethanol, repeatedly.

3.6 ETS

Since high concentration of ethanol is easily vaporized, we thought that vaporization of ethanol occurred from the fermented liquid in a flask. Then, ETS was combined with the fermentation flask and vaporized ethanol was recovered by DW in the bottles as shown in Figure 1. Using ETS, an atmospheric pressure in the flask also remained constant and the yeast cells could maintain high fermentation activities. As a cost-cutting method for the bioethanol production, the repeated ethanol fermentation that combined ETS with the immobilized yeast strain K-7 or C-019 was carried out. We determined amounts of ethanol in two ETS bottles and fermented liquid and considered about the total amount of ethanol formation (Table 2). The maximum ethanol concentrations with which of the strains K-7 and C-19 reached to approximately 85 g/L. The recovery of ethanol by ETS was 2-5 % to total ethanol produced by the fermentation (Table 2).

Table 2: The total amount of ethanol by ETS

	Ethanol in fermented liquid (g)	Ethanol in ETS (g)	ETS/total yield
K-7	8.27 ± 4.5	0.4 ± 0.1	5 %
C-19	8.5 ± 0.25	0.2 ± 0.03	2 %

4. Discussion

In this study, there seemed to be various kinds of inhibitors against the saccharification of materials or yeast activities in the fermentation such as ink, a furfural, a hydroxymethylfurfural(HMF), and a lignin. However, it turned out that ink was separated from paper in the case of saccharification and they did little inhibition in the fermentation. Both the furfural and the HMF are derived by thermal decomposition of glucose, are easy to occur at high temperature and pressure. But since deep physical process was not found to be necessary for our proposal process, these inhibitory substances did not appear to occur. A high condensed glucose liquid above 300 g/L was able to produce in the condensation of saccharified liquid. This liquid , by the fact that the lignin does not exist in the paper shredder scrap, was made with enzymes reaction sufficiently in the saccharification. Furthermore, if biomass including lignin was condensed, glucose is adsorbed to the lignin and it seems the glucose conc. doesn't become high. Therefore, we found there were few inhibitors in this saccharification and fermentation system. Compare the marine derived C-19 strain with referencs strains, fermentation speed was fast per time, superiority in the ethanol fermentation was shown. This might be because marine yeast had osmotic pressure tolerance to high concentration glucose. By agitation fermentation using the C-19 strain, the maximum produced ethanol was 122.5 g/L, whose conc. of Ethanol was easy to vaporize and it is possible to increase total ethanol production with ETS. Since a paper shredder scrap is an industrial waste, the service by the private company has already been built, gathering place is limited with the business district of urban areas, and it is possible to supply it in large quantities, stably. From the experimental results, bioethanol production

from paper shedder scrap was found be at low cost and in high efficiency system. (Heinz et al., 2009) Also reported that bioethanol from waste papers had the greenhouse gas saving potential. That is, the ethanol production using paper shredder scrap by the marine yeast C-19 strain would contribute to Japan's ethanol production.

5. Conclusion

The authors found that glucose of 75 g was produced by only the enzymatic saccharification from the paper of 100 g and the condensed liquid (glucose conc. 270-290 g/L) was prepared for fermentation. The *Saccharomyces cerevisiae* C-19 derived from Tokyo Bay was used for various kinds of fermentation. Furthermore, as a new way to increase the amount of total ethanol, ETS (Ethanol Trap System) was developed. By the agitating fermentation, the maximum ethanol concentration of 12 % (w/v) observed with the C-19 strain. In addition, 8.5 % (w/v) of ethanol concentration was produced by the repeated fermentation .

Acknowledgement

The authors would like to thank Mr. Nobushito Takezawa for his study on the isolation and characterization of the S. cerevisiae strain C-19. For any correspondence, please contact to Dr. Naoto Urano.

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(Received: September 20, 2012; Accepted: December 7, 2012)