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Xylanase extraction from clarified rumen fluid by modified magnetic Nano-particles

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ABSTRACT

In an attempt, Cibacron blue coated Nano magnetic particles were used to extract xylanase enzyme from clarified rumen fluid (as a low cost enzyme source). The mean diameter of Nano-magnetic particles were about 10-20 nm and they were fairly spherical in shape. Adsorption process was studied at different pH values (2-8) and temperatures (6 °C, 22 °C and 45 °C). The highest adsorption was obtained at pH 7.0 (near the isoelectric point of enzyme) and 45 °C ($P < 0.05$). Desorption procedure was carried out using different NaCl concentrations (0.1,0.5 and 1M), and different shaking times (15 and 90 min) at room temperature and pH value of 7.3, which the highest desorption percentage by 61.06 % was achieved at 1M NaCl solution, and 90 minutes of shaking time ($P<0.05$).

1. Introduction

Xylanase is a complex enzyme consists, endo1,4-β-xylanohydrolase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) which degrade xylan polymers to xylooligosaccarides, xylobiose and xylose (Sharma and Kumar, 2013; Nakamura *et al*, 1993). A variety of microorganisms, including bacteria, fungi and yeasts have been reported to produce xylanase (Juturu, 2012). The interest in Xylanase has markedly increased due to its important roles in various industrial processes such as pre-treatment of pulp bleaching, bakery, fruit juice industry, as an additive in animals feeds and nowadays its widely used for bioconversion of lignocellulosic materials for bio-fuel production (Sadiqbutt *et al*, 2008 ; Yan *et al*, 2012). Despite of its importance in industry, the effective usage of xylanase has been restricted by the absence of a reliable enzyme source, therefore finding a suitable and a low cost enzyme source is one of the most important priorities (Miao *et al*, 2012). The ruminants have a complex consortium of different anaerobic microbial groups in their rumen. The rumen anaerobic microorganisms have symbiotic relation with host animal and by producing a wide variety of different enzymes play a major role in food digestion for ruminants. Therefore the rumen fluid is a rich and an appropriate source of microbial enzymes for industrial bio-refineries (Cheng *et al*, 1999; Kitts and Underkofler, 2002). Stanely *et al.* (1956) noted that the clarified rumen fluid (CRF) obtain from slaughtered ruminants had a great fibrolytic property due to presence of xylanase and cellulase enzymes. Zheng *et al.* (2012) reported that, three of the seven possible fibrolytic enzyme sources for enzyme bio-refinery were rumen fluid. Another problem confronted with xylanase production is the difficulties in enzyme extraction and purification techniques, such as chromatography, centrifugation and ultra-filtration, which have some limitations like time consuming, expensive instrumentation, enzyme denaturation, pretreatments and need for skilled operators (Miao *et al,* 2012). Recently nano-magnetic separation have solved many problems regarding the bio-molecules extraction due to their several advantages such as low toxicity, its speed and simplicity (Jourdan *et al*, 2011; Khoshnevisan *et al,* 2011) and more over the high surface to volume ratio that favors the bio-molecules attachments and leads to higher bio-chemical loading per unit mass of particles (Ansari *et al*, 2011). Therefore application of these particles in various biological fields, for example, drug delivery, nucleic acid, enzyme and protein separation and purification is now of high interest (Shamim *et al*, 2008; Yuan *et al*, 2008). The main procedure of this method is to bind nano-magnetic particles with the target bio-molecules to form a complex mixer and subsequently be separated from bulk solution by applying magnetic field. Adsorption of lysozyme enzyme on thermosensitive poly (*N*-isopropylacrylamide) coated nanomagnetic particles at different pH values was investigated by Shamim *et al*. (2007), and the highest adsorption was obtained at pH value of 11. They reported the highest desorption efficiency by 87% was achieved using

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NaH_2PO_4 solution 0.5M. In recent years successful immobilization of fibrolytic enzymes (cellulase and xylanase) on a wide variety of stable support materials and magnetic nano-particles (MNPs) has also been reported (Mestal *et al*, 2003; shah and Guptam, 2008; Tyagi *et al*, 1995). Khoshnevisan *et al.* (2011) reported the successful adsorption of cellulase on superparamagnetic nano particles, which the adsorption capacity and binding efficiency of cellulase was 31mg/g and 95% respectively. Xu *et al.* (2011) for the first time reported successful cellulase immobilization on nano magnetic iron oxide particles via glutaraldehyde binding. In general, immobilized fibrolytic enzymes had a better hydrolysis performance and stabilities (Lio *et al*. 2010). The aim of our study is to separate xylanase enzyme from CRF as a low cost source of enzyme by applying Cibacron blue F3GA coated magnetic nano particles (CBCMNPs). Adsorption procedure was optimized in different temperatures and pH values. Desorption was carried out using 0.1, 0.5 and 1M NaCl solution. To our knowledge this is the first report of xylanase extraction by magnetic CBCMNPs from CRF.

2. Material and methods

2.1. Materials

Iron(II)chloride tetrahydrate, Iron(III)chloride hexahydrate, 3-(trimethoxysilyl)-1-propanathiol, acetic acid, D (+) glucose anhydrous, *N'N*-dimethyl formamide, allyl 2,3-epoxy propyl ether and bovine serum albumin were purchased from Merck Millipore (Germany). Cibacron blue F3GA, 3,5-dinitrosalicylic acid (DNSA), Coomassie Brilliant Blue G-250, Azobisisobutyronitrile (AIBN), xylan (from brichwood) and dialysis tubes with cut-off number 14000 were purchased from Sigma Aldrich (U.S.A), 1,4-dioxon was obtained from Chem-Lab (Belgium), Whatman filter papers was prepared from Munktell (Germany).

2.2. Apparatus

The transmission electron microscope (TEM) images were obtained by using Philips EM208, 100kV (Netherland). Fourier transform infrared (FTIR) spectra were obtained using Thermo Nicolet NEXUS 870 Fourier transform infrared spectroscopy, (USA), and thermogravimetic analysis (TGA) grafts was obtained using Shinko TGA STR-1500 (Korea). Enzyme activity was measured using Varian Cary 50 UV-Vis spectrophotometer (USA).

2.3. Preparation of iron oxide nano particles

Magnetic particle were prepared by chemical co-precipitation (Mahdavian *et al*, 2010). A complete precipitation of nano Fe_3O_4 (4 g) was achieved by dissolving 2.3 g $FeCl_2 .4H_2O$ and 2.3 g $FeCl_3.6H_2O$ in 100 mL distillate water under N_2 atmosphere with vigorous stirring. For three hours the solution was heated to 85 °C while 7.5 mL NH_3 solution (25%) was gently added. The particles were isolated from the solvent by magnetic decantation. The washing decantation procedure was repeated three times (two time with distillate water and the last time with anhydrous ethanol).

2.4. Surface modification of nano-magnetic particles

For surface modification, 4 g of magnetic iron oxide were added to 50 mL dioxin containing 2.5 mL tri-methoxysilyl propanethiol, the mixture was refluxed for 18 hours at 80 °C with vigorous stirring. By magnetic decantation the particles were isolated and then were mixed with 20 mL allyl-2,3-epoxy propyl ether, 50 mL dimethyl formamide and 0.1 g AIBN as an initiator and refluxed for 8 hours at 70 °C under N_2 atmosphere (AIBN was crystallized with ethanol). Finally the previous precipitate were mixed with 1g Cibacron blue F3GA, 50 mL NaCl solution (5%) and 20 mL dimethyl formamide with vigorous stirring at room temperature for six hours. Modified nano-magnetic precipitates were decanted and washed several times with distillated water until the water flowing out turns completely colorless. Bare and coated nano-magnetic particles were characterized by TEM, FTIR and TGA.

2.5. Sample collection and preparation

The rumen fluid samples were collected from 8 slaughtered cows and immediately transported to laboratory and were mix together to create a pool sample. The pool sample was centrifuged ($10000\times g$ for 20 min) and filtered using Whatman filter #4 under suction to remove undigested materials and micro particles to obtain CRF. The obtained CRF was aliquoted to lower volumes (7mL) in tube bottles and were stored in -70 ℃ freezer for a week, and for each experiment six tube bottle were used (as our experimental treatments).

2.6. Determination of xylanase specific activity (Xsa)

Xylanase activity was quantified by measuring the amount of reducing sugar released from the substrate xylan using colorimetric method as described by Miller *et al.*(1957) .One unit of enzyme activity is defined as the amount of enzyme producing 1µmol of xylose equivalents per minute at 50 ℃. Protein concentration was determined according to the procedure described by Bradford (1976) using bovine serum albumin as the standard and Coomassie Brilliant Blue G-250 as the assay reagent . Finally the xylanase specific activity (Xsa) was calculated by the equation below:

1) $\text{Xsa (IU/mg)} = \frac{\text{xylanase activity}\left(\frac{Iu}{ml}\right)}{\text{Protein concentration}\left(\frac{mg}{ml}\right)}$

2.7. Determination of xylanase specific activity of fresh and defrosted CRF

By the equation below, the Xsa of fresh and defrosted CRF was obtained in order to detect the probability of xylanase activity reduction due to the freezing storage and defrosting process. The experiment was performed in six replicates.

2) $E=\frac{DF_C}{F_C}$

Where the DF_C is the Xsa of defrosted CRF and F_C is the Xsa of fresh CRF.

2.8. Adsorption / Desorption of xylanase on surface of modified nano-magnetic particles

Xylanase adsorption was carried out by addition of 0.04g of CBCMNPs into tube bottles containing 7 mL CRF. The tube bottles were shaken for 20 minutes, and experimental parameters such as, different pH values (2-8), and temperatures (6 °C, 22 °C and 45 °C) were optimized in order to obtaining the best sorption. For each experimental parameters six tube bottles (as our replicates) was considered. After optimization, the magnetic field was applied for each experimental tube and the CBCMNPs containing bounded xylanase enzymes were precipitate. The upper supernatant were isolated from precipitates and were used for determination of Xsa of the unbounded xylanase enzyme (A_u) in it. The adsorption efficiency was obtained by the equation below:

3) Adsorption efficiency % = $100 - (\frac{A_u}{A_c} \times 100)$

Where A_u is the xylanase specific activity of the unbounded enzymes in the upper supernatant and A_C is the xylanase specific activity of fresh CRF.

Desorption process was carried out by mixing 7 mL of NaCl solution (0.1, 0.5 and 1 M) with CBCMNPs containing bounded xylanase enzyme from previous stage, in two different shaking times (15 and 90 min) at room temperature. The magnetic field was applied and nano magnetic particles were precipitated. The elutes containing salt and enzyme were dialyzed for 24 hours at 6 °C in order to remove the salt from enzyme solution. The resulting solution containing high dense of enzyme was used to measure the Xas_{des} (A_d) of desorbed enzymes. The desorption efficiency was calculated as:

4) Desorption efficiency % = $[\frac{Ad}{(Ac-Au)} \times 100]$

Where A_d is the xylanase specific activity of desorbed enzymes and ($A_c - A_u$) indicated the extent of enzyme adsorption on CBCMNPs.

3. Statistic

Adsorption data's were analysis by complete randomize design in two stages, the first stage with seven treatments for pH (2-8), the second stage with three treatments for temperature (6 °C, 24 °C and 45 °C), all the stages with six replicates. Desorption data's were also analysis by complete randomize design. The first stage with three treatments for salt concentration (0.1M, 0.5M and 1M), and the second stage with two treatments for shaking time (15 and 90 min) with six replicates. Analysis of variance were performed using software S.P.S.S and the means were compared by Duncan multiple range test.

The statistic model for adsorption and desorption process is as follows:

$J_{ijk} = \mu + t_i + e_{ij}$

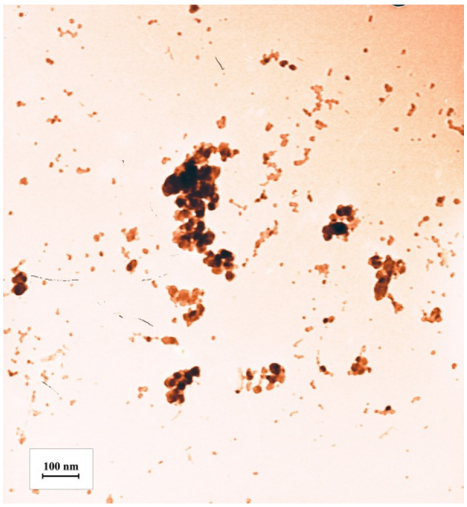
J_{ijk} = Observations
μ = Means
t_i = Effect of treatments
e_{ij} = Effect of error

4. Result and Discussion

4.1 Nano particle size and morphology

TEM micrographs for coated nano particles are shown in Fig 1. The average mean diameter of particles was 10-20 nm. The particles were fairly spherical in shape and completely discrete.

A



B

Fig. 1: TEM micrographs of magnetic nanoparticles. A) Bare Fe_3O_4 particles, B) Coated Fe_3O_4 particles

4.2. Binding efficiency

Fig 2 shows the FTIR spectra for bare and coated iron oxide nano particles. The peaks in 565.7 cm^{-1} and 582.6 cm^{-1} in Fig 2 (a and b) respectively were related to Fe-O in Fe_3O_4 nanoparticles due to the adsorbed water. As shown in Fig 2b, the extra peaks at 1728.4 cm^{-1}, 1144 cm^{-1}, 2996 cm^{-1}, and 1441.8cm^{-1} is due to C=O, C-O, aliphatic OH and CH_3 respectively, which confirmed the chemically modification on nano particles. The TGA was also carried out for both bare and coated CBCNMPs up to 700 °C (Fig 3A, Fig 3B). The mass loss of 5.9% was observed for bare nano particles due to the removal of surface water and water in Fe_3O_4 crystals (Fig. 3A). In other side 23.7% weight lost in CBCMNPs in Fig3b was due to the removal of adsorbed water (5.9%) and decomposition of grafted polymers over inorganic support (17.8%). This result confirms the organic moiety on the inorganic nano particles. In consistent with our results some researchers calculated the maximum amount of decomposed polymers bound to iron oxide nanoparticles about 9% and 12%, and Shamim *et al*.(2008) and Lio *et al*. (2004) respectively(16.29).

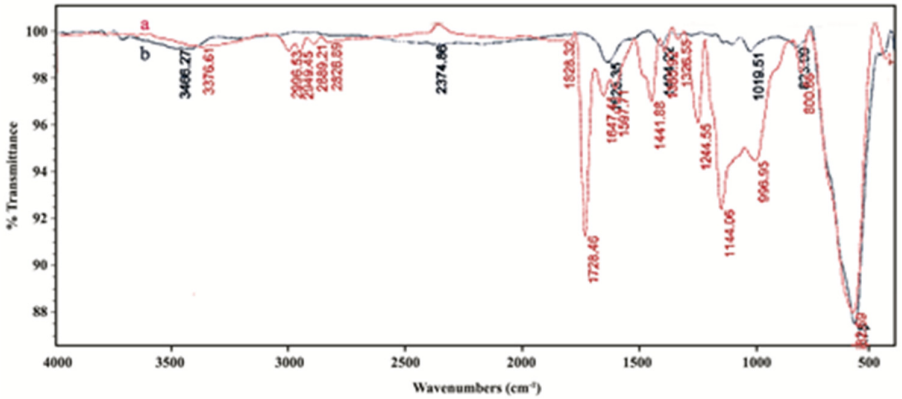
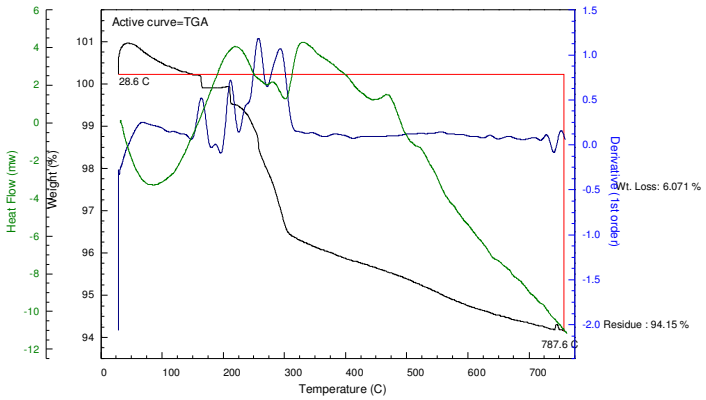
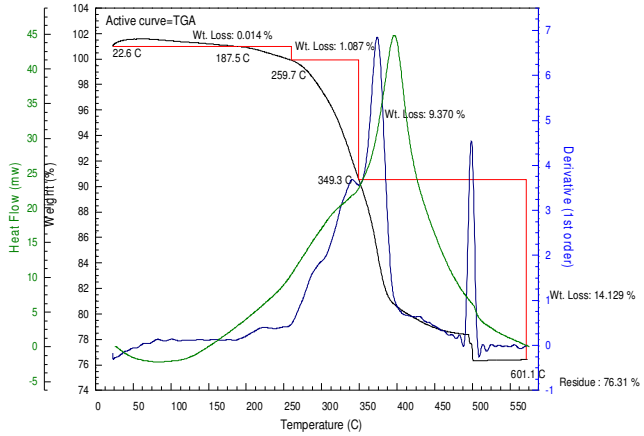


Fig. 2: FTIR spectra of nano-magnetic particles
a) Bare nano magnetic particles b) Coated nano magnetic particles



A



B

Fig. 3: TGA curves. A) Bare and B) Coated magnetic nano magnetic particles

4.3. Freezing and defrosting effects on enzyme activity

The mean result of equation 2 in the section 2.7 was 0.97, which shows a little reduction in xylanase specific activity of defrosted CRF, due to enzymes and proteins deterioration during freezing and defrosting period. Although the xylanase specific activity of defrosted CRF was a bit lower to compare with fresh CRF, the reduction was completely negligible and statistically not significant ($P<0.05$), therefore was not considered in the further experiments. This result is in agreement to the results obtained by Illanes *et al,* (2008). They noted the enzyme solutions can be stored for a short time at -70 ℃ without significant adverse effects on the enzymes structure and efficiency. This result was also in accord with the results of Chamani *et al* (2011). He reported the activities of enzyme solutions stored at -70 ℃ for six weeks had no significant difference compare with the control enzyme solutions ($P<0.05$).

4.4. Effects of pH on xylanase adsorption

The effects of pH on adsorption capacity of xylanase on CBCMNPs were investigated at different pHs (table 1). The highest adsorption efficiency with 48.12 percent was observed at pH value of 7. This result is not unusual because the maximum adsorption of a protein can be obtain in its neutral charge (Shamim *et al*, 2008), and interestingly the isoelectric point of rumen microbial xylanase is between 6 to 7, which charges the side chains of enzyme molecules and favors the adsorption process (Devillard *et al*, 1999; Lee *et al*, 2007; Way *et al*, 2012). The role of dye molecule (Cibacron Blue F3GA) in desorption process is significant. Basar *et al.* (2007) reported, although the isoelectric point of lysozyme enzyme is 11.2, but the highest amount of lysozyme adsorbed on to Cibacron Blue was at pH 7.0 due to its structural nature. In this research the specific interaction (hydrophobic and hydrogen bounding) between xylanase and dye molecule at pH 7, which is completely suitable for adsorption procedure, may results from the active groups on both the Cibacron Blue F3GA and amino acid side chains in xylanase molecules. In the pH value of 6.0 adsorption efficiency is higher than other pH values except for the pH value of 7.0 ($P<0.05$). This result indicates the specific interactions between dye and enzyme molecules still have the power to act properly but its strength is significantly lower than pH 7.0. At pH values lower and higher than 7 the adsorption capacity of xylanase intensely decreases, which the lowest adsorption was estimated at pH value of 8 due to the solubility of nano iron oxide particles in alkaline pH and deterioration of the polymeric ligands on the surface of iron oxide nano particles. At acidic pHs, the ionization state of xylanase and Cibacron Blue (both with positive charges) may create repulsive electrostatic forces, which cause a significant reduction in enzyme adsorption efficiency.

Table1: Xylanase adsorption efficiency at different pHs (at room temperature).

pH	2	3	4	5	6	7	8
Adsorption efficiency %	7.04^{d}	13.49^{c}	16.34^{c}	17.91^{c}	33.42^{b}	48.16^{a}	3.02^{e}

Means with different superscripts are significantly different ($P<0.05$)

4.5. Effects of temperature on adsorption

Fig 4 shows the effects of temperature on adsorption behavior of xylanase on CBCMNPs. By increasing the temperature from 6 ℃ and 25 ℃ to 45 ℃, xylanase adsorption significantly increased from 16.10 % and 48.16 % to 54.22 % respectively. This increment is likely due to higher molecular motility in the higher temperatures, which it's suitable for more enzyme attachments to CBCNMPs. The lowest xylanase adsorption with 16.1% at 6 ℃ was due to the statistic state of enzyme at this temperature, which it's not favor for enzyme attachments.

4.6. Desorption of xylanase from CBCNMPs

To study the desorption of xylanase form CBCMNPs, NaCl solution was used as an desorption agent to decrease interaction forces between enzyme and adsorbent, by promoting the ionic strength in the medium (Garcia *et al*, 2008; Sutkeviciute *et al*, 2008; Odabasi *et al*, 2004). Therefore desorption procedure was carried out at different NaCl concentrations (0.1M, 0.5 M and 1M), and two different shaking times (15 and 90 minutes) at room temperature. The results from Fig (5) shows in 15 minutes of shaking time, increases of NaCl concentration form 0.1M to 0.5M had no significant effect on desorption efficiency with 18.1% and 20.23% respectively ($P<0.05$), while by increasing the salt concentration to 1M the increment of desorption efficiency (29.5%) was meaningful ($P<0.05$). It means with different superscripts are significantly different ($P<0.05$).

CBCMNPs in different NaCl concentrations and shaking times. It means with different superscripts are significantly different ($P<0.05$).

In this study the effects of NaCl concentration was completely obvious, which in 90 minutes of shaking time by increasing the salt concentration from 0.1M to 0.5M and 1M the desorption efficiency significantly increased from 22.91%, to 31.12% and 61.06% respectively ($P<0.05$). In NaCl solution, the

salt ions have higher charges and lower ionic radii relative to protein (enzyme) molecules, therefore the salt ions can compete with the enzyme molecules for binding to CBCMNP surface.

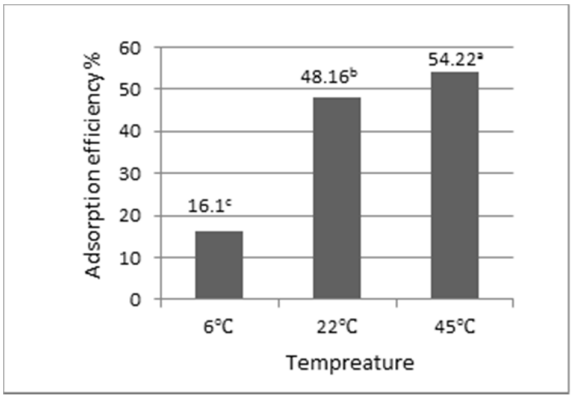


Fig. 4: Effects of temperature on adsorption efficiency of xylanase on CBCMNPs (at pH value of 7.0)

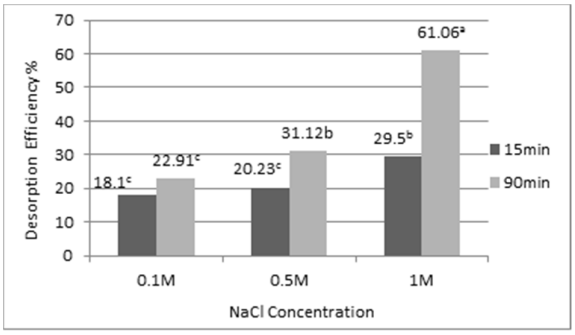


Fig. 5: Xylanase desorption efficiency from

Salt ions causes the enzyme molecules are expelled from the CBCMNP and the enzyme molecules replaced by salt ions. The shaking time also had direct effect on desorption efficiency too, which by increasing the shaking time from 15 to 90 min especially in 0.5M and 1M salt solution, desorption efficiency increased from 20.23% to 31.12% and from 29.5% to 61.06% respectively ($P<0.05$). This result indicate by increasing the shaking time the salt ions have enough time to eliminate the enzyme molecules from CBCMNPs and have enough time to contact with dye molecules.

5. Conclusion

Nano magnetic iron oxide particles were prepared and modified by chemical co-precipitation method. The morphology and modification of the particles were confirmed using TEM, FTIR and TGA respectively. The best adsorption efficiency (54.22%) was achieved at pH 7.0 and 45 ℃. Highest desorption efficiency with 61.06% was achieved by NaCl solution (1M) in 90 minutes of shaking time. The obtained results indicate that Cibacron Blue F3GA coated magnetic nano particles probably can be effective appliance for xylanase extraction from clarified rumen fluid by optimizing adsorption/desorption condition.

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