ISSN No:-2456-2165

Biodiesel Production using Neem Oil with Lipase from *Pseudomonas aeruginosa* TEN01

Saravanan R[1]
Research scholar,
Department of Mechanical Engineering,
J.J College Of Engineering and Technology. ,
Trichy -620009, Tamilnadu,India

Tamil Elakkiya V[1]*
Assistant Professor,
Department of Biotechnology,
Anna University, BIT Campus,
Trichy-620024, Tamilnadu, India

Sivakumar D.B.[2]
Assistant Professor,
Department Of Mechanical Engineering,
Anna University, BIT Campus,
Trichy- 620024, Tamilnadu, India

Abstract:- Biodiesel is defined as the mono-alkyl esters of long-chain fatty acids derivative from vegetable oils or animal fats using a catalyst. Neem oil is non-edible oil and it is an effective alternative source for the production of biodiesel. But till now, heterogeneous chemical catalysts are used to convert the neem oil to biodiesel. Here, we focused on the higher production of biodiesel from neem oil using microbial lipase as a catalyst. Lipase was produced from Pseudomonas aeruginosa TEN01. The productivity of the organism was examined for free cell and whole-cell immobilization methods. Both free and immobilized cells produced lipase activity was 4.6 U/ml.min-1 on 48h. Produced lipase was partially purified by ammonium sulphate precipitation and dialysis. Partially purified lipase-catalyzed the reaction of methyl ester formation from triglycerides present in the neem oil. The primary characterization was done by TLC and the biodiesel was confirmed through flash point and fire point test.

Keywords:- *Biodiesel; Neem Oil; Lipase; TLC; Flash Point Test.*

I. INTRODUCTION

Production of Biodiesel (Fatty acids alkyl esters, FAAEs) with cheap and renewable resources is becoming the hot topic of every country's policy agenda [1]. Due to limited energy reserves and the increasing environmental pressure on green-house gases coming from fossil fuels, the US predicted that the requirement of fuel could be 98.3 million barrels/ day in 2016 and it would be 118 million barrels/day in 2030. Biodiesel has drawn its attention due to its non-toxicity, bio-degradable, renewable source of fuel and energy with significantly lower exhaust emissions of particulate matter and greenhouse gases such as CO, CO2, and SO2 [2]. Azadirachta indica, also known as Neem, Nimtree, is native to India and the Indian subcontinent including Nepal, Pakistan, Bangladesh and Sri Lanka. It is typically grown in tropical and semi-tropical regions. Neem oil also contains several sterols, including campesterol, beta-sitosterol, stigmasterol, etc. A higher amount of triglycerides are present in the neem oil. Therefore, these triglycerides can be converted into glycerol and methyl esters by lipase or other catalysts through the trans-esterification process. Lipase is ubiquitous, serine hydrolase enzyme, and it hydrolyzes triacylglycerols to glycerol and free fatty acids. It is activated only when it absorbed the oil-water interface.

Many lipases are active in organic solvents where they catalyze several useful reactions including esterification, trans-esterification, region selective acylation of glycols and enthols and synthesis of peptides and other chemicals.

II. MATERIALS AND METHODS

- *Extraction of neem oil*

The seeds are collected and it was de-coated and it was dried under shadow condition for 2-3 days. And it was kept overnight on oven for 60°C to remove the moisture. About 5L of neem oil was collected from 2-3 kg of seeds using expeller. The oil is stored at room temperature for the further processes.

- *Bacterial Strain revival*

0.5ml of stored *Pseudomonas aeruginosa* TEN01 was thawed at 37°C and it was inoculated into Nutrient broth and all the flasks were incubated for 72 hours at 37°C in order to cell revival.

- *Preparation of enrichment media*

Revived *Pseudomonas aeruginosa* TEN01 strain was streaked onto the nutrient agar plate. After overnight incubation, the single colonies were inoculated into 50 ml of specified enrichment media for Lipase and incubated for 10h for 125rpm at 37°C. It contained Peptone (0.1%), Yeast extract (0.18%), Ammonium sulphate (0.35%), Potassium di hydrogen phosphate (0.3%), Sodium chloride (0.25%), Magnesium sulphate (0.1%), Sunflower oil (5%), Toluene (1%).

- *Preparation of production media*

2% (v/v) of enriched cells were introduced production media for lipase. The production media included, Yeast extract (2%), Beef extract (1%), Glucose (0.5%), sun

flower oil (0.5%), Dipotassium hydrogen phosphate (0.1%), Magnesium sulphate (0.05%). The growth of the organism was observed by at 590nm at regular intervals of 2-3h.

➢ *Lipase activity*

Lipase production was confirmed by three methods. (i) Tributyrin assay, (ii) Tween 20 plate assay and (iii) Phenol red assay.

➢ *Tributyrin Assay*

Tributyrin assay carried out by disc diffusion assay. The plate was incubated at 37°C for 48h and the plate was observed for zone of clearance. *Bacillus subtilis* was made as the positive control and sterilized Production media was made as a negative control.

➢ *Tween 20 plate assay*

A simple method to calculate the lipase activity of the organism. 1% of Tween-20 was mixed with nutrient agar. Organisms taken at different time intervals were placed onto the agar plate by means of the disc and it was incubated for 24h to observe the extent of growth.

➢ *Phenol red assay*

The cell free supernatant was collected at 8000rpm at 15min on different time intervals were plated on the agar plate containing phenol red (0.01%), agar (2%) and Tween 20 (2%). The zone of clearance was also observed.

➢ *Estimation of Lipase*

The lipase activity was checked by titration method. Add 2 ml of 0.1M phosphate buffer (pH 7.0), 1 ml of oil and 1 ml of crude extract was incubated at 40°C for 30 minutes. The reaction was stopped by adding 5 ml ethanol and it was titrated against 0.1 N NaOH using phenolphthalein as indicator. The appearance of permanent pale pink colour was the end point.

Lipase activity was calculated using the formula,

$$\text{Lipase activity} = \frac{\text{Volume of alkali consumed X Normality of NaOH}}{\text{Time of incubation (min) X Volume of enzyme solution}}$$

➢ *Whole cell immobilization*

Cells were entrapped with sodium alginate method. Sodium alginate (3% w/v) was prepared and it was added with the equal volume of culture. The mixture was introduced drop wise into 0.2 M $CaCl_2$ solution taken in the petridish and kept for curing for 2 hours. They were preserved in 0.9% saline solution and kept in a refrigerator for the further processes.

➢ *Purification of Lipase*

➢ *Ammonium sulphate precipitation*

After the incubation period, the culture was centrifuged at 12,000 rpm for 20 min at 4ºC. An enzyme preparation was obtained by precipitation with 30-60% ammonium sulphate fractionation. At the end of fractionation the mixture was centrifuged at 10,000×g for 30 minutes at 4°C and the pellet was collected and resuspended in small volume of 0.05 M phosphate buffer (pH 7.0) and checked for the enzyme activity.

➢ *Dialysis*

The dialysis membrane (10KDa) was pretreated with 2% $NaHCO_3$ and 10 mM EDTA. Then the sample was loaded in the pretreated membrane and it was placed in the stirring for 48h at 4ºC.

➢ *Biodiesel production*

5g of oil and rational amount of enzyme, methanol and water were added and placed for the reaction at 45°C for 25h. The calculated methanol was added in three subsequent steps at 0, 3, and 6h of the complete reaction. The water and enzyme concentration were 8.43% and 7.19% respectively. Methanol was added three times the volume with that of oil. The upper layer was collected and it was washed with warm water for several times and heated to 130°C to remove water content, left methanol and other impurities.

➢ *Primary characterization by TLC*

Thin Layer Chromatography is one of the methods to characterize biodiesel. The mixture of Chloroform: Methanol: Calcium oxide in the ratio of 6:3.5:0.8 was used as mobile phase and iodine solution was used as a spraying solution. The brown colour spot indicates the presence of methyl esters.

➢ *Analysis of Biodiesel – Flash point and Fire point*

About 50ml of sample used for analysis of flash point and fire point. The flash point and fire point were to be found out in order to check with the biodiesel standards.

III. RESULT AND DISCUSSION

➢ *Time course study of biomass*

The growth profile for the organism was observed at regular intervals. The organism showed higher growth on 48th hour. The growth curve is depicted as follows:

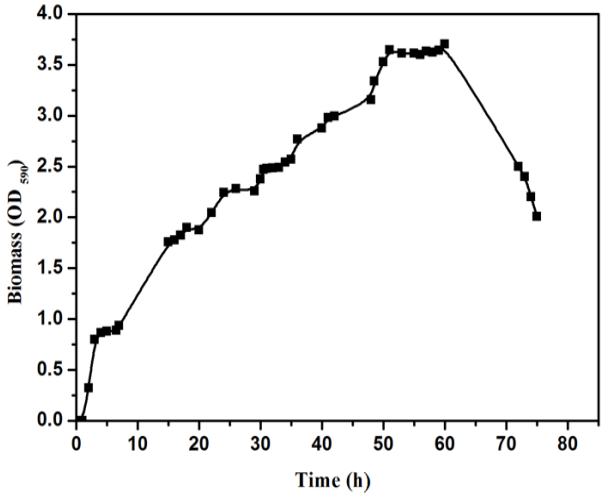

Fig 1:- Time course study of Biomass

➢ *Lipase activity*

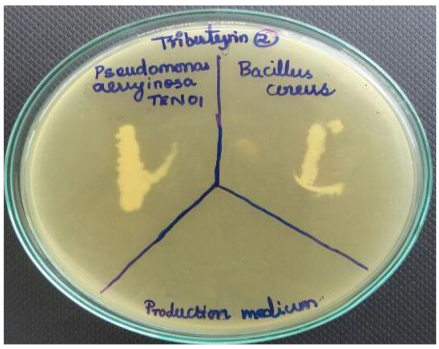

Fig 2:- Tributyrin assay

The figure (3) indicated that the maximal growth of organism was found on 48th hour sample as there was the hydrolysis of tween 20 was more on 48h. Specifically, pH reaches 6.5 which indicates the production of fattyacids. The figure (d) indicated that the change in colour of phenol red revealed the lipase activity.

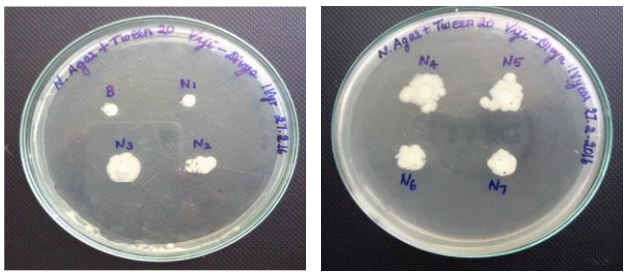

Fig 3:- Tween 20 plate assay

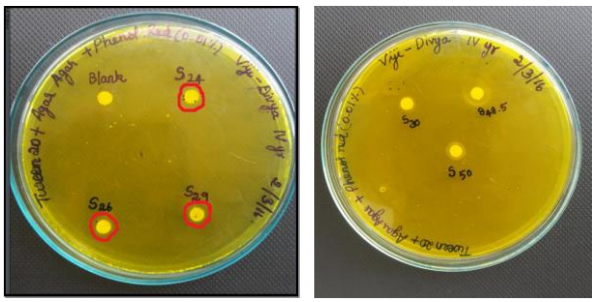

Fig 4:- Phenol red clearing zone assay

➢ *Estimation of Lipase*

The lipase activity was calculated using the specific formula and maximum lipase activity was confirmed to be on 48th hour.

Fig 5:- Estimation of lipase

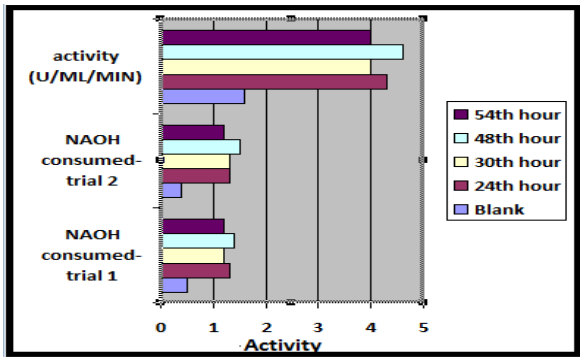

Table 1:- Lipase activity of extracellular free cell lipase

➢ *Whole cell immobilization*

The total bead counting were about 221. The beads were stored in 0.9% saline in refrigerator. The higher enzyme activity (4.4 U/ml/min) was observed in 48h culture and the beads are reused second time the same activity was maintained.

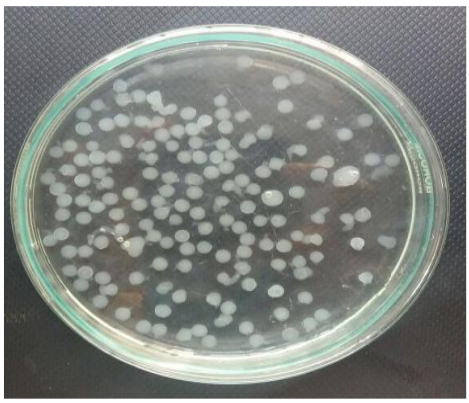

Fig 6:- Immobilization of whole cells

➢ *Purification of enzyme Ammonium sulphate precipitation*

The supernatant of extracellular free cell lipase with lipase activity of 4.3 U/ml/min was used as crude enzyme and subjected to partial purification by ammonium sulphate precipitation in fraction of 30-60%. The supernatant of immobilized cell lipase with 4.4 U/ml/min activity was used ad crude enzyme and subjected to the purification.

➢ *Dialysis*

The crude sample was dialyzed using 10kDa molecular cut-off membrane. The salt contents are eliminated from the crude sample and the dialyzed sample had 4.6 U/ml/min enzyme activities. The lipase from the immobilized cell has showed lesser lipase activity than that of lipase from free cell. But, the immobilized cell lipase has much more reusability than free cell lipase.

➢ *Biodiesel production by volume ratio 3:1*

The biodiesel production was carried on the basis of volume ratio 3:1 of methanol and neem oil. The neem oil was taken as 50ml and the methanol was 150 ml and the enzyme concentration was 7.19% and the reaction time was about 24h. The top biodiesel was removing the unreacted methanol (as the boiling point of methanol is only 65°C).

Then, the remaining biodiesel was stored. The retention factor for the plate was calculated as 0.62.

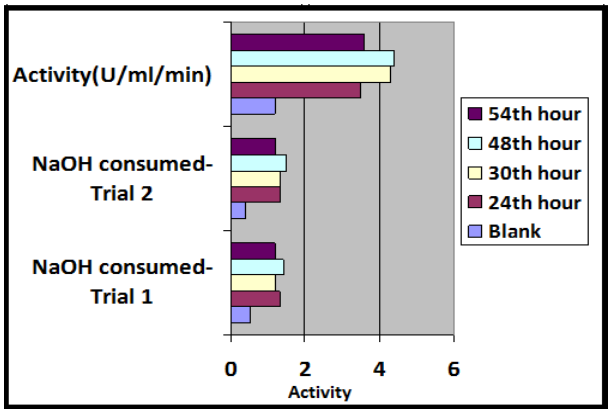

Table 2:- Lipase activity of extracellular immobilized cell lipase

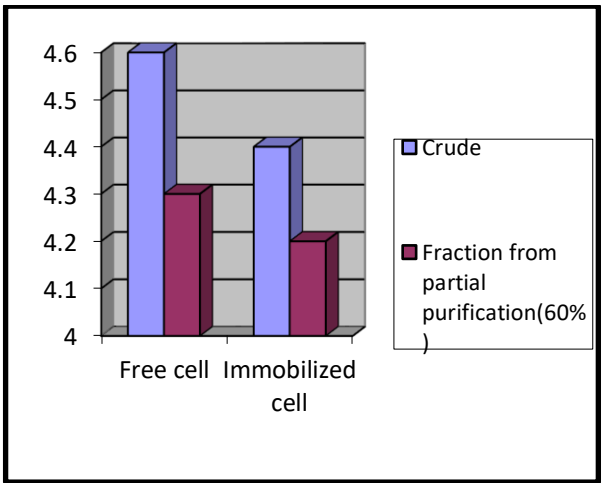

Table 3:- Lipase activity of extracellular free cell lipase and immobilized cell lipase after Precipitation

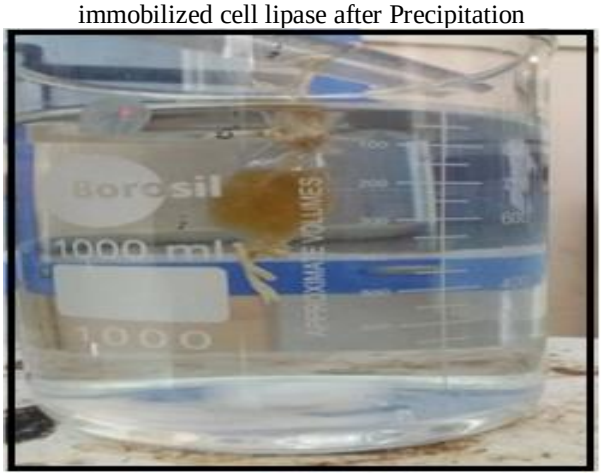

Fig 4:- Dialysis of Lipase

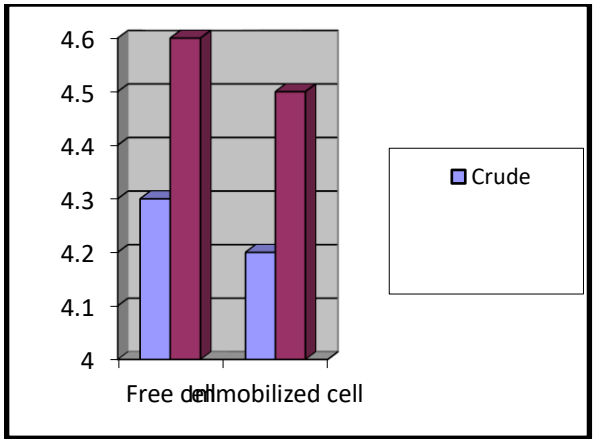

Table 5:- Lipase activity of the xtracellular free cell lipase and immobilized cell lipase after dialysis

Fig 7:- Biodiesel production

➢ *Biodiesel Analysis: Flash point and Fire point*

The biodiesel produced from neem oil using lipase was confirmed by flash and fire point. The biodiesel fired at 135◦C and flashed at 132◦C.

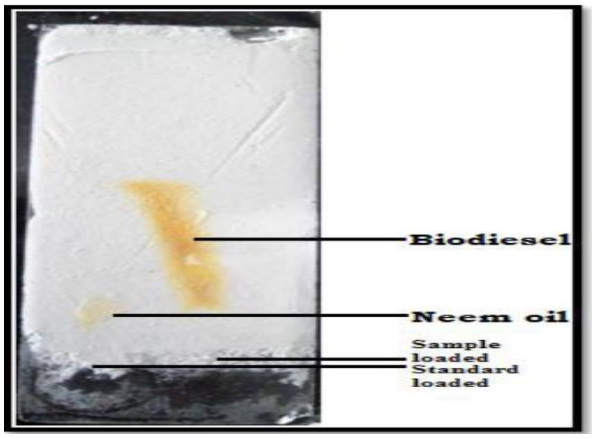

Fig 8:- TLC of Biodiesel

➢ *Biodiesel Analysis: Flash point and Fire point*

The biodiesel produced from neem oil using lipase was confirmed by flash and fire point. The biodiesel fired at 140ºC and flashed at 132ºC.

IV. CONCLUSION

In this study, Lipase from *Pseudomonas aeruginosa* TEN01 was produced and the higher lipase activity was obtained on 48h. Compared with free cell lipase, the immobilized cell enzyme activity was lower but it has the reusable ability. The partially purified lipase activity was similar with the crude lipase activity and produced lipase was made as a catalyst for the production of biodiesel using neem oil and methanol as substrates. From the result of TLC, the experimental Rf value was calculated. Therefore, the methyl ester formation is primarily characterized and it was confirmed by Flash and fire point tests.

REFERENCES

[1]. Anya Uzo Anya, Nwobia Noelle chioma, Ofoegbu Obinna – Optimized reduction of free fatty acid content on Neem seed oil, for Biodiesel production – J.Basic.Appl. Chem, Vol 2 (2012)-page-21-28
[2]. Barnwal B K, Sharma M P – prospects of biodiesel production from vegetable oils in India, 2005 – Renewable and sustainable energy reviews – Volume 9 –Pages- 363-378
[3]. Bull O S; Obunwo CC 2014 - "Bio-Diesel Production from Oil of Orange (Citrus Sinensis) Peels as Feedstock" - Journal of Application and science environmental management.Vol.18 page.371-374.
[4]. Colette breuil, D.B. Shindler, J.S. Sijher and D. J. Kushner – Stimulation of lipase production during Bacterial growth on alkanes – Journal of Bacteriology (1978) – volume 133 - page – 601-606
[5]. Elizabeth Funmilayo Aransiola1. 2013. "Lipase Catalyzed Ethanolysis of Jatropha Oil for Biodiesel Production" - Energy and Environment Research. Vol. 3, page. 1
[6]. Fukuda S, Hama S, Thamalampudi S, Noda H - whole Whole-cell biocatalysts for biodiesel fuel production, 2008- Trends in biotechnologyVolume 26 –Pages-668-673 7. H. Muthu, V. SathyaSelvabala, T. K. Varathachary, D. Kirupha Selvaraj,J.
[7]. H. Muthu, V. SathyaSelvabala, T. K. Varathachary, D. Kirupha Selvaraj,J. Nandagopal2 and S. Subramanian1. 2010. "Synthesis of biodiesel from neem oil using sulfated zirconia via tranesterification"- Brazilian Journal of Chemical Engineering Vol. 27, No. 04, page. 601 – 608.
[8]. L C meher, Vidya S.S. Dharmagadda, S.N. Naik – Optimization of alkali-catalyzed transesterification of *Pongamia pinnata* oil for production of Biodiesel – Biosource Technology 97(2006)- page-1392-1397
[9]. Mohd yusof A.Samad, C. Nyonya A. Razak, Abu Bakar Salleh, W.M. Zin Wan Yunus, Kamaruzamam Ampon, Mahiran Basri – A plate assay for primary screening of lipase activity – journal of Microbiological methods 9 (1989) – page -51-56
[10]. Murugesan, D. Subramanian, A. Avinash, N. Nedunchezhian - Quantitative and qualitative analysis of biodiesel – an in-depth study – International Journal of Ambient Energy -36:1- pages-19-30- 2014
[11]. Mustafa Canakci, 2007 – The potential of restaurant waste lipids as biodiesel feedstocks – Bioresource Technology, volume 98, page- 183-190
[12]. Olugbenga Olufemi Awolu and Stephen KolawoleLayokun. 2013. "Optimization of two-step transesterification production of biodiesel from neem (*Azadirachta indica*) oil "- international journal of enery and environmental engineering.
[13]. Sylvester C. Izah and Elijah I. Ohimain. 2013 - "The challenge of biodiesel production from oil palm feedstock in Nigeria"- Greener Journal of Biological Sciences. Vol. 3 (1), page. 001-012.
[14]. Pradeep Kr Vishnoi, Rahul Bharti , P.C.Tewari, V.K. Gupta, "Production of neem oil ethyl ester (NOEE) or neem oil biodiesel by two step esterification of filtered neem oil"-MATRIX Academic International Online Journal Of Engineering And Technology.
[15]. QingchunJi, Sujing Xiao, Bingfang He, Xiaoning Liu.2010. "Purification and characterization of an organic solvent-tolerant lipase from *Pseudomonas aeruginosa* LX1 and its application for biodiesel production"- Journal of Molecular Catalysis B: Enzymatic.page.264-269.
[16]. Rahul Mukherjee, John marshall High School, Rochester, Minnesota – macroalgae as an alternative source of biodiesel: A study on native algae from the great lakes for Industrial utilization
[17]. Rohit Sharma, Yusuf Chisti, Uttam Chand Banerjee – Production, purification, characterization and applications of lipases – Biotechnology Advances 19 (2001) – 627-662
[18]. Samuel J.Allen, Lisa S. Ott, 2012- HPLC method for rapidly following biodiesel fuel transesterification reaction progress using a core-shell column- Analytical and bioanalytical chemistry-Volume 404 – Pages- 267-272.
[19]. Sensoz S, Angin D, Yorgun S- Influence of particle size on the pyrolysis of rapeseed (*Brassica napus* L.): fuel properties of bio-oil – Biomass and Bioenergy-Volume 19-pages-271-279
[20]. Shaika, Sarika Chaturveda, Aradhana Khare- Isolation and optimization of the Extracellular Lipase under submerged Fermentation – Intrenational journal of Applied Engineering Research- Vol. 10 No.35-2015
[21]. Shruthi H.Heroor and S.D. Rahul Bharadwaj – Production of Bio-fuel from Crude neem oil and its Performance – International journal of Environmental engineering and Management, Volume 4 (2013) page-425-432