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Bio-Tech & Genetics

Highlights

- Fixed-Dome Biodigester
- Isozyme Profile of Esterases
- Engineered Bovine Serum
- Nitrosomonas and Nitrobactor

Discovering Thoughts, Inventing Future

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Bio-Tech & Genetics

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Characterization of Biogas Produced from Rice Husks and Algae using a Metal Fixed-Dome Biodigester

By Ezekoye, V. A, Onah, D. U, Offor, P. O. & B. A. Ezekoye
University of Nigeria, Nigeria

Abstract- Rice husks and algae plant substrates were success- fully used to produce biogas. A metal fixed-dome biodigester (bioreactor) was used for the characterization of the biogas generated from these plants wastes. A total of 35 kg of slurry (sludge) made from 5 kg of rice husks and 30 kg of algae were mixed in water in the ratio of 1:6 and fed into the biodigester (bioreactor). The digestion of slurry was undertaken in batch-type anaerobic digestion and mesophilic temperatures range at 29.00°C – 33.45°C. For over period of 75 days, the cumulative biogas produced from the wastes was 156.25 litres. The percentage of the methane component of produced biogas was 52.3% .The biogas from the seeded rice husk was combustible on the 45th day.

Keywords: biogas, anaerobic digestion, mesophilic, characterization, combustible.

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CHARACTERIZATION OF BIOGAS PRODUCED FROM RICE HUSKS AND ALGAE USING A METAL IXED-DOM BIODIGSTER

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Characterization of Biogas Produced from Rice Husks and Algae using a Metal Fixed-Dome Biodigester

Ezekoye, V. A [α], Onah, D. U [σ], Offor, P. O. [ρ] & B. A. Ezekoye [ω]

Abstract- Rice husks and algae plant substrates were successfully used to produce biogas. A metal fixed-dome biodigester (bioreactor) was used for the characterization of the biogas generated from these plants wastes. A total of 35 kg of slurry (sludge) made from 5 kg of rice husks and 30 kg of algae were mixed in water in the ratio of 1:6 and fed into the biodigester (bioreactor). The digestion of slurry was undertaken in batch-type anaerobic digestion and mesophilic temperatures range at 29.00ºC – 33.45ºC. For over period of 75 days, the cumulative biogas produced from the wastes was 156.25 litres. The percentage of the methane component of produced biogas was 52.3% .The biogas from the seeded rice husk was combustible on the 45th day.

Keywords: biogas, anaerobic digestion, mesophilic, characterization, combustible.

I. Introduction

About one third of the world's population have little or no access to modern energy services. Majority of these people are living in poverty. The acute symptoms of this poverty, as well as its chronic causes, are critically linked in many ways to today's patterns of energy production and use. Renewable energy resources (biogas) are available and replenishable. Biogas is formed solely through the activity of bacteria. Bacteria have a temperature range in which they are most productive in term of production rates, growth rates and substrate degradation performance. Several groups of bacteria involved in anaerobic digestion have different temperature optimum. This results in two main temperature ranges at which digestion usually can be performed optimally and most economically. These ranges are 30-45.00 °C called the mesophilic range and 45-70 °C called the thermophilic range (BTG, 2003; Ezeonu *et al*., 2005).

Biogas is made up of methane, carbon dioxide, nitrogen, hydrogen sulphide, ammonia and water vapour. The percentage composition of these gases varies depending on the substrates and the optimum conditions of biogas production (Jenangi, 2002, Philip and Itodo, 2002). Temperature and nutrient addition (Seeding) were one of the most important factors that affect biogas production. The rate of bacteriological methane production increases with digester temperature, retention time and with the percentage of total solid/volatile solid in the slurry (Werner *et al*., 1987, Dioha et al., 2006, Kalia and Singh, 1996). This is because temperature and addition of inocula affects the enzymatic activities of the microorganisms (anaerobic) responsible for the conversion of organic materials into biogas (Kepler, 2006, Maurya *et al*., 1994).

To guarantee optimum biogas production, it is very important to mix various raw materials in accordance with carbon to nitrogen (C/N) ratio requirements of the fermentation. The C/N ratio reflects the relative proportions of these two elements in the digester. Carbon (in form of carbohydrates) and nitrogen (as protein, nitrates, ammonia, etc) are the chief nutrients for anaerobic bacteria. There are two major differences between the digested and undigested products. More volatile nitrogen is contained in anaerobically digested manure and nutrients are more uniformly distributed in anaerobically digested manure. The temperature of mesophilic fermentation is preferred worldwide because: it is easy to maintain the digester at this temperature. Mesophilic bacteria are more stable than thermophilic bacteria. They produced high quality sludge (Chawla, 1969; Bardiya and Gaur, 1997).

In addition to the production of biogas as a form of energy, the use of plant waste as raw materials for biogas production is also a plus for sanitation technology. In this study plant waste (Rice husk and Algae) has been used as raw material for biogas production. The biogas produced was stored under pressure in cylinders using compressor for domestic uses.This paper aim at producing combustible biogas from biomass (rice husk and algae), to study rate of production of biogas, to study biodegradation of rice husk and algae and to investigate the effect of fermentation temperature on the production rate of biogas from organic waste.

II. Materials and Methods

a) Materials

The rice husks and algae collected from community were used for the experiment. The digester

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used was fixed-dome type. The basic parts of the digester are shown and labelled in the schematic diagram in figure 2 below.

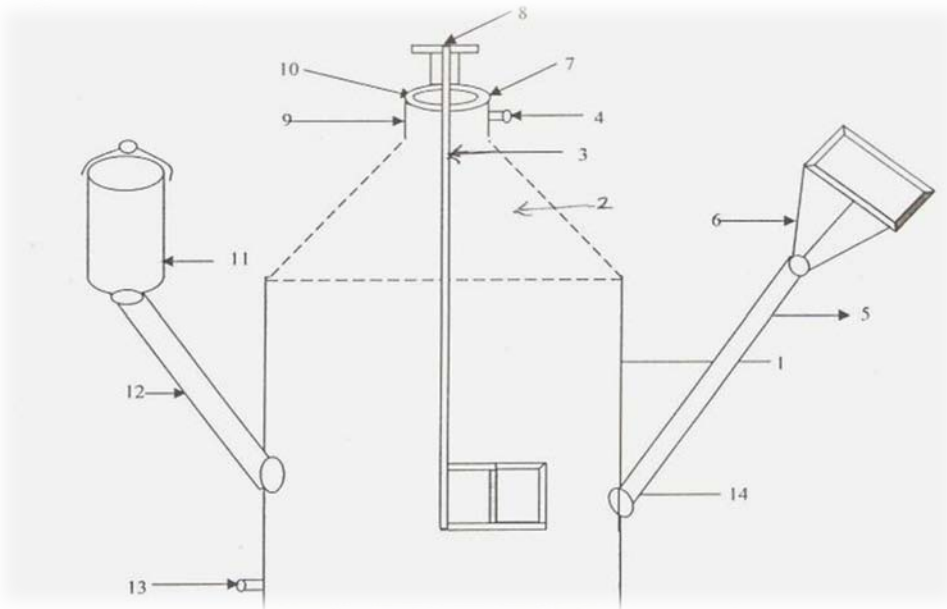

Figure 1 : Schematic Diagram of the biogas digester

Part no. = part name:

1 = Fermentation unit,
3 = stirrer shaft,
5 = slurry inlet pipe,
7 = bolts hole,
9 = short metal neck,
11 = cylindrical outlet mouth,
13 = Nipple and socket,
2 = gas storage unit,
4 = gas outlet pipe,
6 = funnel-like mouth,
8 = stirrer handle,
10 = metal rim/ring,
12 = slurry pipe,
14 = shaft blade

III. Methods

a) *Apparatus used and testing of Digester*

Figure 2 : Fixed-dome biogas digester (NCERD UNN)

The figure above is the photograph of the fixed-dome biogas digester used for digestion of rice husk. The rice husk and algae were treated in anaerobic sequencing batch reactor or digester whose capacity was 0.971 m^3 and operated at 29.00 °C as their maximum ambient temperature and 35.45 °C as their maximum slurry temperature.

The digester was tested for leakage and its ability to absorb radiant energy (heat). Leakage arising from any part of the digester will inhibit microbial activities of methanogenes. The microbes responsible for anaerobic digestion of organic waste to produce mahtane are adversely affected by the presence of oxygen.

Biogas digestion is a microbial process and therefore requires the maintenance of suitable growth condition for biogas-producing bacteria. These operating parameters like nutrient, an optimum temperature, pH and other environmental factors are vital for the activities of living bacteria. Only when these conditions are met will the normal bacterial activity and subsequent gas production is assured (Yadvika, 2004; Song *et al*., 2004).

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In order to maintain an optimum mesophilic temperature range, i.e. temperature within the range of 30°C to 40°C, the walls (inside and outside) of the digester were painted with a dull black colour paint to enhance the absorption of heat for proper metabolic activities of methane-producing micro- organisms.

b) Experimental Procedure for Rice Husk and Algae

The digestion of waste was undertaken by a metal fixed-dome type of anaerobic digester. The fixed-dome digester was accessed using rice husk and algae from a sewage pond. The rice husk and the algae water were mixed in the ratio of 1:6. Hence 5kg of rice husk and 30kg of algae water were mixed together thereby summing up to 35kg of content of the digester. After charging of digester, daily ambient and slurry temperatures were recorded as well as daily volume of gas yielded for a about 75days retention time (RT) .See table 1.

Using the built-in stirrer, the slurry was stirred regularly to distribute and maintain uniformity of temperature and thus, the thickening and caking of scum was prevented. Stirring did not only distribute temperature, it also ensured even distribution of bacteria and at the same time improved surface contact of the waste with the anaerobic bacteria, thereby speeding up biochemical reaction of fermentation.

The volume of the daily gas produced was measured by method of downward displacement of water using a graduated twenty-litre jerry can under atmospheric pressure. The combustibility of the gas produced was checked severally until the gas became combustible.

Table 1 : Days of flammability and total biogas produced.

Waste	Flammable time (day)	Retention time (Days)	Total biogas produced (L)	Temp.(°C)	Mixing Ratio
Rice husk/Algae	45	75	156.25	29.00-35.45	1:6

IV. Results

a) Proximate Analysis

Some samples of the waste were taken to Department of Crop Science laboratory, University of Nigeria Nsukka for proximate analysis on the waste during the course of the research at Exhibition Centre of National Centre for Energy Research Development. About 0.04kg of the waste was collected and analyzed in the laboratory for determination of concentration of various components of the slurry before and after digestion (see table 2).

In the present study the C/N ratio of 6:1was notice at the initial stage which decreases with the passage of substrate decomposition. The result corroborated with the study of Philip and Itodo (Philip and Itodo, 2002).

In the experimental sets, initial pH value of substrate was found to be 6.4.As the decomposition preceded, the pH value gradually increased up to 8.0, while the final value was recorded as 7.1, indicating the stability of organic matter. Earlier studies have indicated that pH range of 5.5-9.0 was suitable for microbial decomposition of organic materials, while the compositing process was most effective at pH values between 6.5 and 8.0 (Kalia and Singh,1996; Philip and Itodo, 2002).

Table 2 : Proximate Analysis before and after digestion

Parameters	Rice Husk/Algae	
	Before digestion Quantity (%)	After digestion Quantity (%)
Ash	3.50	0.14
Carbohydrate	17.38	8.17
Carbon	12.64	1.2..37
Fats	2.00	1.82
Fibre	2.43	Trace
Moisture	72.50	80.36
Nitrogen	2.19	9.63
Ph	6.40 (not in %)	7.10 (not in %)
Phosphorus	7.75ppm	6.483ppm
Volatile Solid	6.30mg/l	2.82mg/l
Total Solid	8.00mg/l	4.32mg/l

b) Flammability of the gas

Three days after charging of the digester, biochemical reaction due to microbial activities gave rise to production of biogas observed from rise in water level at the outlet of the digester resulting from downward displacement due to the pressure built inside the digester by the biogas. Several combustibility tests were run using a special burner designed for biogas

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combustion. The first test was run 10days after production of gas began and the test proved negative. This test was repeated at intervals of about 7times until positive result was obtained on the 45th day. The gas burnt with a blue fame, producing no soothes.

c) Measurement of Volume of Biogas Produced

The gas produced was measured under atmospheric pressure using the method of downward displacement of water contained in an inverted calibrated transparent 20 litre jerry can. The calibration of the can was expressed in litres. This jerry can was inverted in wide plastic basin with the free end of the hose connected to the digester passing through the brim of the can. The volume of the biogas was determined by measuring the volume of water displaced by the gas as the tap on the digester was turned on. Daily volume of the gas was recorded. For the period of about 62 days an appreciable production of gas was recorded, a total volume of approximately 156.25 litres of gas, an average of 2.52 litres per day (Arnell and Vallin, 2007; Wikipedia for free encyclopedia,2011; Yadvika, 2004).

Table 3 : Percentage of the component of biogas from Rice husk/Algae wastes using Orsat Apparatus

Waste	Carbon dioxide (CO_2) (%)	Hydrogen sulphide H_2S (%)	Carbon monoxide (CO) (%)	Methane and other components
Rice husk/Algae	30.7	2.1	9.9	52.3

V. Discussion

It has been noted in the literature that temperature and retention time are among the parameters that influence anaerobic fermentation of organic matter. From figure 3, it can be observed that from retention time (RT) interval between 4 days and 34 days, the rate of production of biogas was almost constant. Between about 38 days and 55 days, the rate of generation of the gas was increased. Within this time interval, the gas was tested and it became flammable. Also, figure 3 shows daily variation of volume of gas with retention time. The maximum daily gas produced was recorded during fermentation, it was about 6.50 litres.

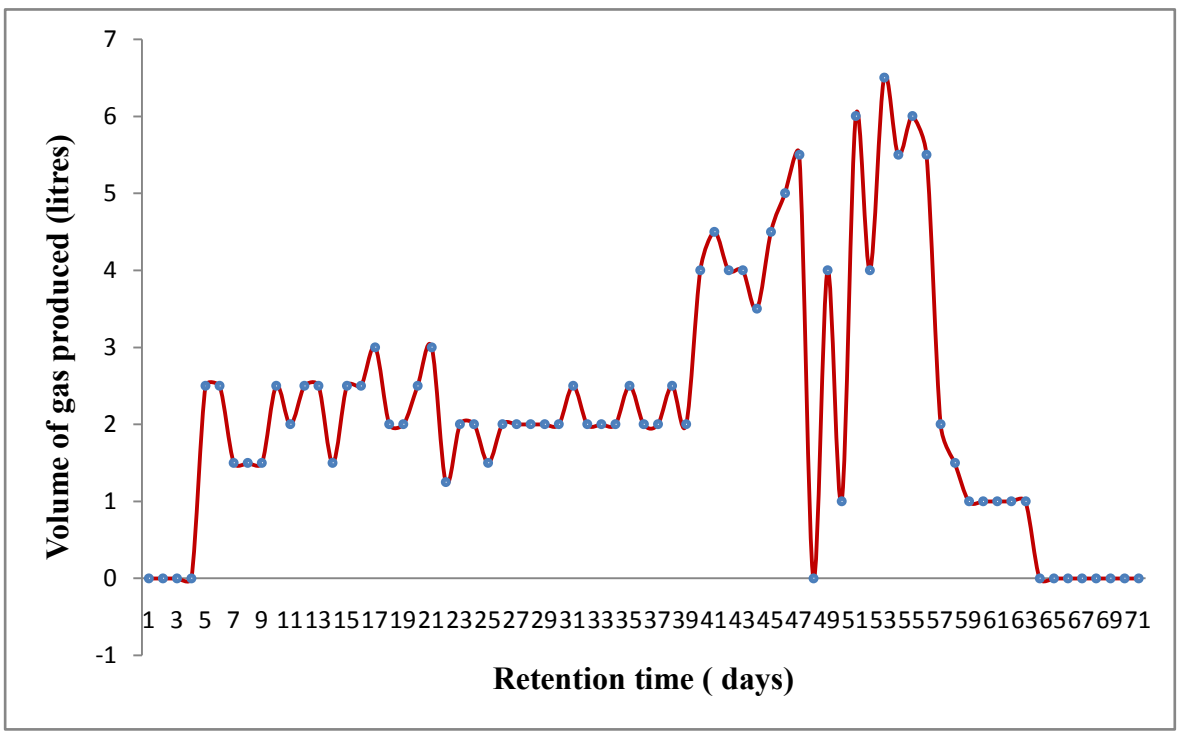

Figure 3 : Daily volume of gas produced

Figures 4 and 5 also show variation of ambient temperature and slurry temperature with retention time respectively.

From the study (see table 1), the slurry average temperature was measured to be about 33.45°C. This temperature was within the mesophillic temperature range. On the other hand, the ambient has an average of about 29.00°C. Also figure 4 gives the distribution relationship between slurry and ambient temperature which shows that the increase in slurry temperature was proportional to the ambient temperature.

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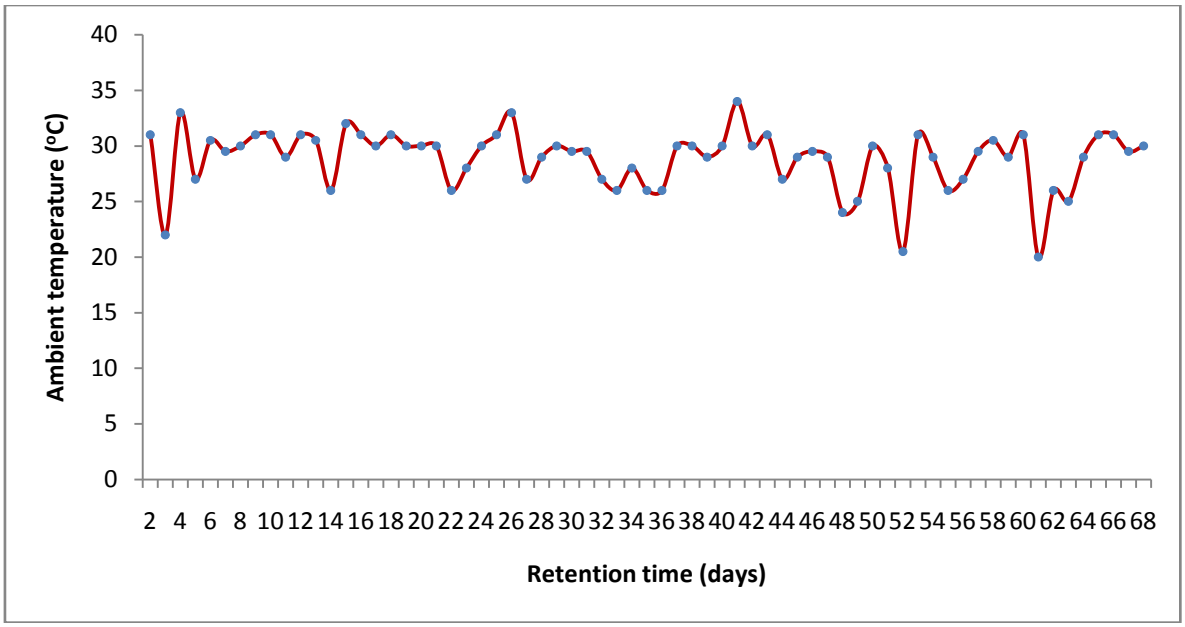

Figure 4 : Daily change in ambient temperature during fermentation

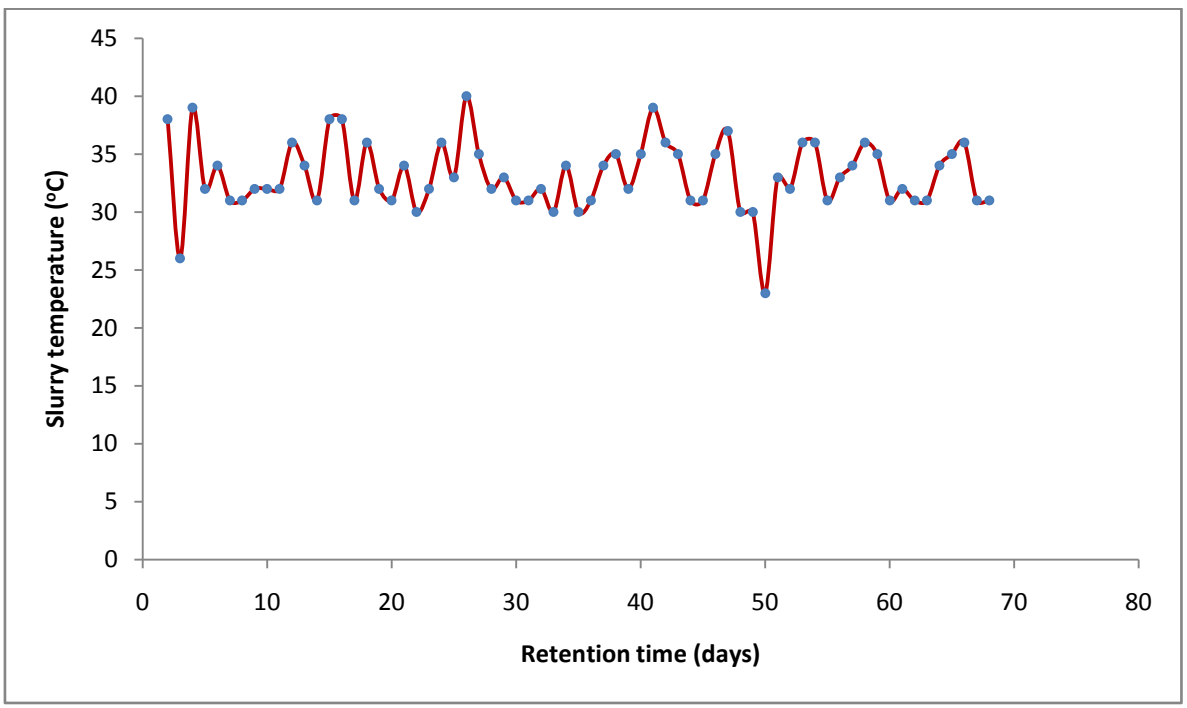

Figure 5 : Daily changes in slurry temperature during fermentation

Results obtained from the study have indicated inoculum age influence in cumulative gas and average daily gas production. Seeding the digester with algae collected from sewage pond as an inoculum generated a total biogas of 156.25 litres within a retention time of 75 days. The average daily gas produced was calculated to be 2.52 litres .Percentage analysis of the component of biogas produced from Rich husk/Algae wastes was done using Orsat Apparatus (see table 3). Table 2 shows the Proximate Analysis of Rice husk/ Algae before and after digestion.

The percentage the methane component of biogas produced was 52.3% .The mean weekly temperature was found to be in the range of (29.00–33.45^{0}C) which was high mesophilic range of temperature. Figure 6 show graph of cumulative volume of biogas produced for 75 days.

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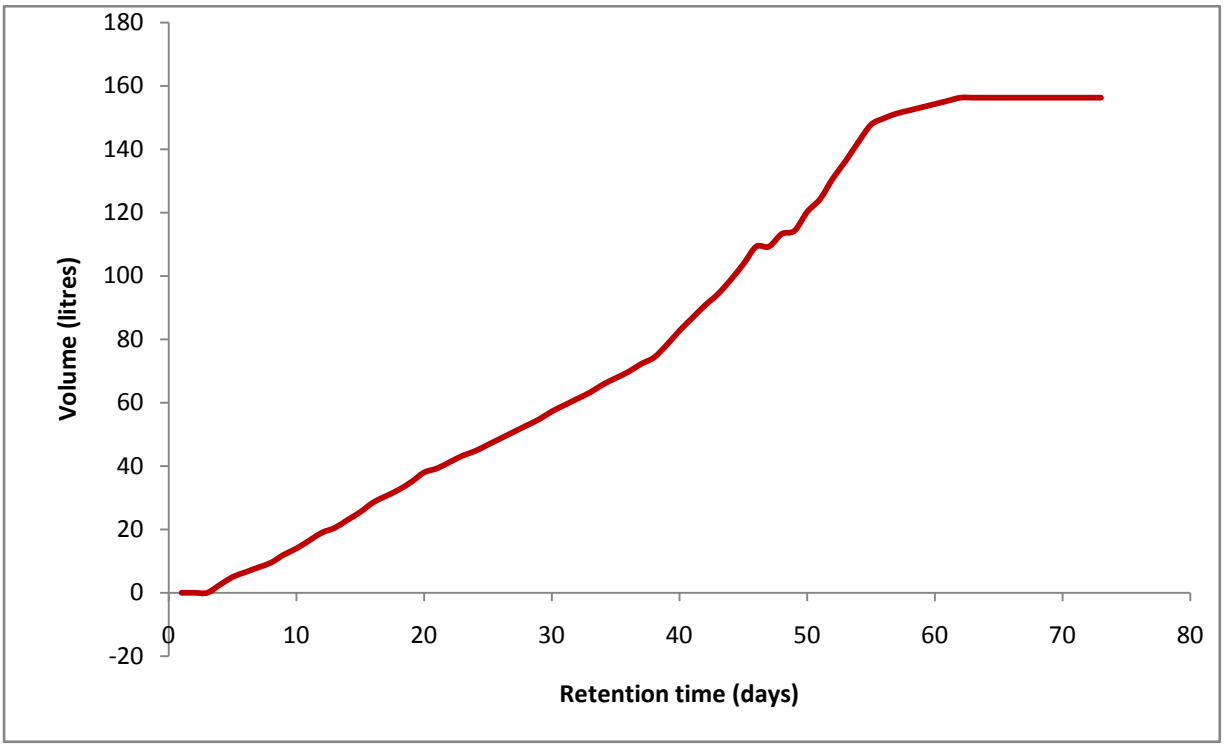

Figure 6 : Variation of cumulative volume of biogas against retention time

The two temperatures fall within the mesophilic range. Figure 7 illustrates the regression temperature between the slurry and ambient temperature. For Rice husk and Algae from seawage pond substitute the values of x = 33.0°C for maximum ambient temperature and x =22.0°C of the minimum ambient temperature on the prediction equation Y=0.62x +15.24. The predicted maximum and minimum slurry temperatures were 40.00° and 26.00°C. The equation R^2=0.313 shows the coefficient of determination which explains proportion of two variables. For example in Rice husk and Algae from seawage R^2 = 0.313, it means that the relationship between the maximum and minimum temperatures of ambient and slurry temperatures was 31.3%. The co-efficient of correlation was deduced from the coefficient of determination R^2, for the waste, it was 0.559. The correlation coefficient between slurry temperature and ambient temperature was r =0.56. This was used to support the claim that the increase in the ambient temperature could be attributed to the increase in slurry temperature (Philip and Itodo, 2002; Goodger, 1980).

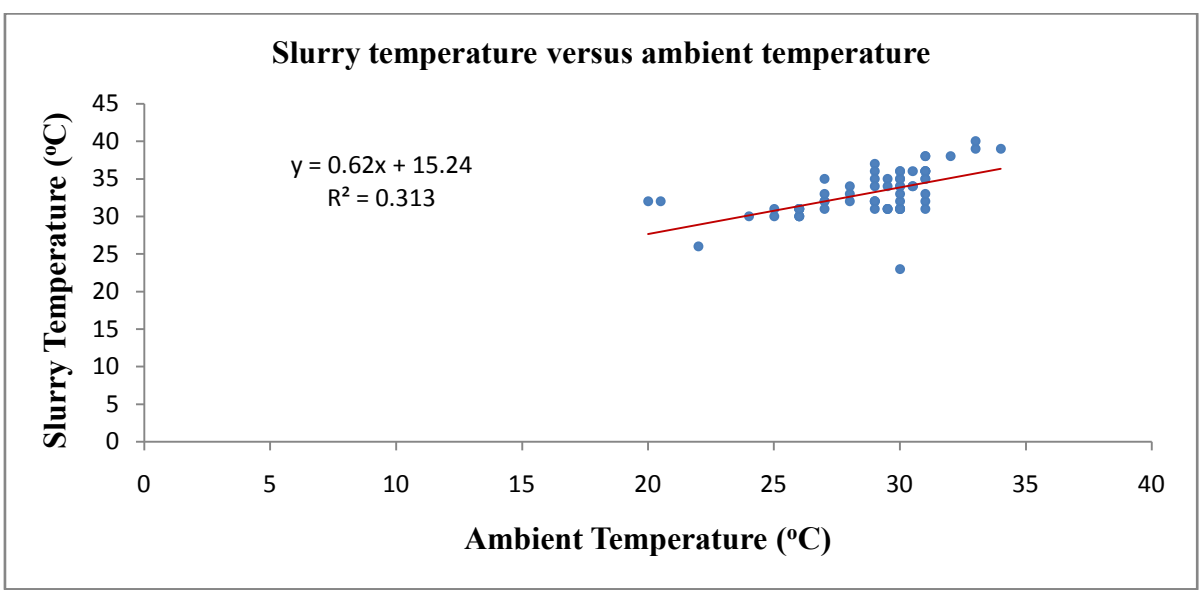

Figure 7 : Slurry temperature versus ambient temperature

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Table 2 shows the Proximate Analysis of Rice husk/Algae before and after digestion

VI. Environmental Benefits and Applications of Biogas

Biogas system designed to process animal and human excreta are expected to contribute to a cleaner and healthy environment in the following ways: elimination of smoke reduces the incidence of lung and eye diseases, especially among village inhabitants; improved rural sanitation due to systematic collection and processing of animal dung and human excreta. This also leads to reduction in water-borne diseases caused by lack of sanitation; and aids to prevent deforestation and consequently soil erosion, flood and climatic effect.

Also, well-functioning biogas systems can yield a whole range of benefits for their users, the society and the environment in general through: production of energy (heat, light, electricity); transformation of organic waste into high quality biofertilizer; improvement of hygienic conditions through reduction of pathogens, worm eggs and flies; and reduction of workload, mainly for women, in firewood collection and cooking.

Environmental advantages through protection of soil, water, air and woody vegetation: micro-economical benefits through energy and fertilizer substitution, additional income sources and increasing yields of animal husbandry and agriculture; and macro-economical benefits through decentralized energy generation, import substitution and environmental protection.

VII. Conclusion

The result of this research has shown that many of the microorganisms associated with the fermentation of rice husk and algae from sewage pond originated from the inoculum and substrate used. Also since the population of the microbes in the digester was increased by addition of the inoculum, there was fierce competition for the limited substrate, and the intensity of this competition depends on the net population of the microbes in the digester. This is the determinant factor for the retention time of the substrate as well as the quantity of the biogas produced. The addition of inoculum to rice husk was found to enhance gas production. It was also found to be influential especially in specific gas production, cumulative gas production and percentage degradation of solid particles. An inoculum of between two to three weeks of age could be used as a seeding agent for starting up biogas digesters.

VIII. Recommendation

Algae from seawage pond are recommended as the best seeding material so far. Also a 1:6 ratio (feedstock to water) is therefore recommended for optimal digestion of Rice husk and Algae from seawage pond. To encourage the use of biogas requires an awareness of the potential users of the process.

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Isozyme Profile of Esterases in Backcrosses of *Catla Catla* (Ham.) and *Labeo Rohita* (Ham.)

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Abstract- Under captive conditions of ponds, reservoirs and other stagnant aquatic bodies in Indian peninsula, the major carps viz. - Catla catla (Ham) and Labeo rohita (Ham) have their unique and equal importance contributing lion shares to freshwater aquaculture. With an intention to improve the genetic architecture of these carps, their backcross gener-ations were developed in Central Agricultural Research Institute, Port Blair, South Andaman to establish some of the desired morphometric characters such as small and narrow head of rohu as well as deep, broad body of catla in their backcross progenies using the technique of induce breeding. To reveal the hereditary trend, the esterase profile was developed and the isozyme marker indicated more genetic proximity of backcross progenies with rohu than catla.

Keywords: *catla catla, labeo rohita, backcross, esterase.*

GJSFR-G Classification : *FOR Code: 290101*

ISOZYMEPROFILEOFESTERASESINBACKCROSSESOFCATLACATLAHAM.ANDLABEOROHITAHAM

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Isozyme Profile of Esterases in Backcrosses of *Catla Catla* (Ham.) and *Labeo Rohita* (Ham.)

Subodh Kumar Tripathy [α] & Niranjan Sarangi [σ]

Abstract- Under captive conditions of ponds, reservoirs and other stagnant aquatic bodies in Indian peninsula, the major carps viz. - *Catla catla* (Ham) and *Labeo rohita* (Ham) have their unique and equal importance contributing lion shares to freshwater aquaculture. With an intention to improve the genetic architecture of these carps, their backcross generations were developed in Central Agricultural Research Institute, Port Blair, South Andaman to establish some of the desired morphometric characters such as small and narrow head of rohu as well as deep, broad body of catla in their backcross progenies using the technique of induce breeding. To reveal the hereditary trend, the esterase profile was developed and the isozyme marker indicated more genetic proximity of backcross progenies with rohu than catla.

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I. Introduction

Revolutions in agriculture and animal sciences were the boons for civilization. Running parallel to these achievements, fisheries also created a significant impact on the life style of common man throughout the globe. Aquaculture gained a status of industry from home stead activities in late nineties in Indian subcontinent. The broad arena of aquaculture encompasses captive fishery with tremendous potential in food production. Lannan *et al*. (1989) indicated the global demographic trend for urgent need of fisheries for human health irrespective of per capita animal fat consumption. As per Manna (1989), fishes serve the best source of animal proteins where people prefer fish to meat. The concept of harnessing animal proteins from aquatic organisms created fascination towards aquaculture, but it still remains a dream to bring much-awaited 'blue revolution' even though it has been described as a low-energy costing practice requiring less input for protein yield.

More than half of the population in developing countries get at least 40% of animal proteins, from fish (F.A.O., 2000) and India contributed around 3.6 million tons to global freshwater fish production in 2009. Indian aquaculture is highly promising and has grown over six and half fold in the last four decades with freshwater aquaculture contributing over 95% of the total freshwater fisheries production. The two Indian Major Carps viz. - *Catla catla* (Ham.) and *Labeo rohita* (Ham.) contributing lion share to aqua farming are the most imperative for freshwater aquaculture in Indian peninsula being widely adopted throughout the sub-continent due to their culture potential, availability and market demand. But it is always realized that, the larger head per unit body weight of catla is a major disadvantage (Basavaraju *et al*. 1995) so far as edible flesh content per unit body mass is concerned. Rohu is scattered naturally in various river systems of India (Jhingran and Pullin, 1985) and is one of the world's principal aquaculture species in terms of production (Hulata, 2001) as per Islam and Alam (2004). Therefore, a good amalgamation of deep catla type body and narrow rohu type head is always a notion of considerable importance for aquaculture requiring apt hybridization. As the existing variations within a group of individuals provide ready opportunity towards preferred progenitors for selection, the idea of genetic evaluation on the line of varietal improvement by backcrossing is the core of the experimental design for the present study. A need was felt to improve the species/variety through introgression of some desirable gene(s) or quantitative trait(s) as hinted earlier by Sinha and Khan (1989) as well as Padhi and Mandal (1996) through backcrossing. Backcrossing is a well-known and long established breeding scheme where a characteristic is introgressed from a donor parent into the genomic background of a recurrent parent. Sinha and Khan (1989) discussed under inter-generic hybridization that, F_1 of catla x rohu may be allowed *inter se* breeding to develop F_2 progenies or backcrossed with parental species to establish new characters. Selection in backcross programmes is used to either improve the genetic value of plant and animal populations or fine map quantitative trait loci. Both cases are helpful in our understanding of the genetic bases of quantitative traits variation (Hospital, 2005). Backcrossing isolates a gene or chromosomal region in a different genetic background (the genetic background of the recurrent parent), it helps to dissect the genetic architecture of quantitative traits.

The relevance of biochemical tool for revealing the usefulness of the genetic architecture in an organism including fishes can be well understood since last two and half decades of research in various part of the globe from Hames and Rickwood (1990), Oliver *et*

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al. (1991), Van der Bank *et al*. (1992), Buth (1993), Sarangi and Mandal (1996), Skibinski and Ward (1998), Arai and Mukaino (1998), Sarangi *et al.* (1999), Sarangi and Mandal (2000) and Sarangi *et al*. (2002). As per Holmes and Whitt (1970), there were a large number of loci responsible for a complex group of enzymes like esterases in vertebrates. In the context of genetic evaluation of the backcross progenies, it was realized that, esterase marker as a potential biochemical genetic tool must be employed to gain some view in to the hereditary trend. Biochemical genetics involving isozyme/allozyme analysis as an important genetic marker was extensively used to characterize plant genetic resources (Tanskley and Orton, 1983 and Heun *et al*., 1994). Tanskley and Orton (1983) reviewed the application of esterase as markers in plants where as Weeden *et al*. (1984) discussed the importance of esterase as biochemical marker in bean yellow mosaic virus resistance. Stuber *et al.* (1987) used this technique to manipulate quantitatively determined characters as per Powell (1992). Takada (2004) reported the usefulness of esterase as genetic markers to study the composition and its dynamics in *M. persica* populations.

Reddy and Laxmipathy (1990) reported 30 esterase bands in various tissues of rohu with wide variations in partition coefficient (Rf) values between individuals from adjacent collection sites of the same river system indicating a high degree of genetic differences. Mucous of rohu showed three esterase bands in two regions (Padhi and Khuda-Buksh, 1990). As per Chatterjee (1994) esterases are the most extensively surveyed isozymes in animal models. Among the Indian Major Carps and other cyprinid species in South Asia, many species-specific markers have been detected, using esterase (Gopalakrishnan, 1997).

Keeping this in view, the present study was undertaken to perform breeding of different backcross generations of catla and rohu with an aim to develop a new variety/strain with desirable traits. Biochemical analysis through esterase profiling was done for genetic evaluation of the developed progenies. As no such study in backcross generations of catla and rohu with respect to their parental generations are available, it may prove useful in future related to their genetics.

II. Materials and Methods

a) *Production of backcross progenies*

Backcross generations of catla and rohu were developed in Central Agricultural Research Institute (CARI), Port Blair, South Andaman through systematic breeding approach from the founder stocks of the parental generations developed from the seeds procured from the hatchery unit of Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, Odisha during 1987 as a part of hatchery development programme in CARI (Tripathy *et al*., 2010). The mating design followed was mostly 1:1 brooders for experimental purpose where as it was 2:3 or 1:3 (male: female) for farm requirement to meet the demand of local farmers and entrepreneurs every year. The F1 hybrids were developed by crossing catla female and rohu male and were designated as C x R or C R. Subsequently, the F2 hybrids were produced from inter se breeding of F1 progenies (CR x CR), the first backcross generation or B1 was produced from F1 and catla (CR x C), where as B2 from F1 and rohu (CR x R). Hybridization of B1 and rohu resulted in B1R (CR x C) x R and BC1F2 were from inter se breeding of B1 (CR x C) x (CR x C). The breeds were maintained in separate pools and ponds without allowing any mix up. Side by side various tissue samples were collected and preserved for future use.

b) *The specimen*

A total of 15 individuals from each generation of carps like catla, rohu, F2, B1R and BC1F2 ranging in size from 30-40 mm were selected for the purpose. The skeletal muscles, heart, liver and kidneys were collected with utmost care at sub-normal temperature arranged in ice packed boxes to prevent degradation of tissues and stored immediately after collection under refrigeration for future use. In case of BC1F2 some whole specimens were selected randomly ranging from 7.0 to 8.0 mm at hatchling stage and crushed.

c) *Esterase profiling*

Esterase profiling was done (EST-1*, EST-2*, EST-3*, EST-4*, EST-5*, EC: 3.1.1.) as per Sarangi and Mandal (1996) following Abersold *et al.* (1987). The followed experimental protocol was for vertical slab gel electrophoresis (5% native polyacrylamide gel) in discontinuous buffer system (Rechardson et al. 1986) using Bio-Rad made mini electrophoretic apparatus (Mini Protein-II, Catalogue no 165) at 4°C with running voltage of 250 V for 2 hours. The staining recipe was of Shaw and Prasad (1970), using substrate as 1% α, β napthyl acetate made of α- napthyl acetate 1 g and β napthyl acetate 1 g dissolved in Acetone 50 ml and H2O 50 ml. Oliver et al. (1991) reported that, napthol acetates might not be suitable for exhibiting sites of low esterase activity as those substrates were hydrolyzed more slowly than napthyl acetate. The stain was made of Fast Blue RR 100 mg dissolved in 10 ml of 0.5 M TRIS of p H 7.1 and 87 ml of 1% α, β napthyl acetate and 3 ml of H2O. The gel was incubated at room temperature until blue band appeared and then the gel was washed and fixed.

The co dominant isozyme bands were assigned different codes like aa, AA, Aa, BB and bb for homozygous conditions and AB, Ab, aB, bB for heterozygous conditions of loci. The data based on isozyme polymorphism of esterase markers were analyzed and the genetic distance matrix was

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constructed by POPGENE-32. The dendogram was constructed by employing the statistical soft ware based on original formulae of Nei (1972, 1978) for similarity index and genetic distance correlation by Unweighted Paired Group Method with Arithmetic Averages (UPGMA). It calculated the genetic distances and identities based on the formulae of pair-wise similarities (SAB) as per Lynch (1990) originally based on Nei (1972) and Nei (1978) for similarities index, using the data for SAB = 2 NAB/(NA+NB), where NAB is the number of common bands between individuals A and B, and NA and NB are the total number of bands possessed by individuals A and B. The mean pair-wise similarity S was computed as S = ΣSAB/n where it is the arithmetic mean of all S values. As per Lynch (1990), the variance of S was calculated as V (S) = 2S (1-S)(2-S)/ N(4-S). N stands for average number of isozyme bands per individual.

III. Results

a) Esterase profile

The result of esterase profiling is presented in table 1, 2, 3 and Figure 1 as well as the photographic plates 1, 2 and 3 summarizing the information on its polymorphism for comparison giving the values of genetic distance correlation, probability of genetic distance correlation and genetic distance matrix of co-ancestry identity and Nei's genetic distance respectively.

Correlation of genetic distance (Theta) based on the polymorphism in the band pattern of various loci for esterase (Table 1) for catla indicates highest distance correlation with rohu (0.57), which is considered obvious due to their difference at generic level. Catla is lowest correlated to BC_1F_2 then other generations. The distance of catla with F_2, B_1R and BC_1F_2 are 0.42, 0.51 and 0.38 respectively. When the distance of various generations with rohu is considered, F_2 shows highest correlation with respect to genetic distance (0.06). Lowest value is observed with BC_1F_2 (0.02). B_1R generation is more distantly correlated to catla (0.51) than rohu (0.03) and with BC_1F_2 (0.02) and F_2 (0.12). The BC_1F_2 backcross generation shows more distance with catla (0.38) followed by F_2 (0.05) and with rohu and B_1R (0.02). The correlation for genetic distance between both the back cross generation i.e. B_1R and BC_1F_2 is 0.21. The probability of genetic distance correlation (Theta P) for esterase for all the loci analyzed shows highest value (0.22).

The Table 2 presents the genetic distance matrix of co ancestry identity for esterase based on the loci analyzed for polymorphism. That of catla based on genetic distance matrix is highest with rohu (0.50) and lowest with BC_1F_2 (0.25). The co ancestry of catla and B1R is more (0.42) than that with F_2 (0.31). Co ancestry of rohu is highest with catla and lowest with B_1R (0.02). Those of rohu with BC_1F_2 and F_2 are 0.03 and 0.07 respectively. The B_1R shows highest co ancestry identity with catla (0.42) and lowest with rohu and BC_1F_2 (0.02). That with F_2, is 0.03. The BC_1F_2 shows the co ancestry identity in ascending order with F_2 (0.002), B_1R (0.022), rohu (0.03) and catla (0.25).

Table 3 presents the genetic distance values as per Nei (1972) calculated by the software POPGENE-32 based on the polymorphism for esterase loci in various generations of catla and rohu. Catla shows Nei's distance of 0.21, 0.22, 0.29 and 0.42 with F_2, BC_1F_2, B_1R and rohu respectively in ascending order. Highest value of rohu is with catla followed by BC_1F_2 (0.05), F_2 (0.03) and B_1R (0.033). The F_2 shows maximum distance with catla (0.21) followed by rohu (0.03), B_1R (0.025) and BC_1F_2 (0.02). B_1R shows maximum distance with catla (0.29) and minimum with F_2 (0.025). This generation is at a distance of 0.03 with rohu and 0.02 with BC_1F_2. The BC_1F_2 generation shows maximum genetic distance with catla (0.22) followed by rohu (0.05), B_1R (0.02) and F_2 (0.02).

Based on the genetic distance matrix of esterase, the constructed genetic distance tree i.e. the dendogram in the figure 1 shows two groups of carps clustering rohu along with other carps generations and catla in a separate branch.

IV. Discussion

So far as breeding backcross generations of these Indian Major Carps is concerned, it is a new concept in terms of the products derived for genetics of both the carps. As both the species interbreed among each other, it is easy to induce them for inter-generic breeding but the hindrance of the technique is that both have long generation periods of 2-3 years, hence their care and maintenance is highly time consuming, tedious and labor intensive. Therefore backcrossing is not followed usually in catla or rohu. But it is a frequently followed conventional technique in various plant models with short generation period for different classical genetic analyses. This type of crossing is generally attempted in plants important to horticulture and in some lower group of animals like worms, insects, some fishes and mammals with short generation period. Although aquaculture organisms differ from agricultural mammals and birds in several important ways (e.g. higher fecundity and smaller post-embryonic size), the principles of selective breeding can also be applied to their genetic improvement. The success of breeding backcross progenies of catla and rohu and their genetic evaluation to find out the heredity may be attributed to nature itself, which accepted intra-generic hybridization resulting in viable hybrids which is due to their compatibilities at chromosomal level (Zhang and Reddy, 1990).

Historically, biochemical markers such as allozymes/isozymes are used for genetics where the

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product of a gene rather than the DNA sequence itself is examined (polymorphism is detected in the form of bands at different positions in electrophoresis for enzyme activity. Powell (1992) wrote on the importance of esterase as isozyme marker in plant genomics that, those were the most widely used protein markers in breeding programmes. Kober *et al*. (2004) cited various examples of writing on the importance of esterase analysis in some legume symbiont bacteria.

Simonsen et al. (2004) reported allozyme studies in three species of Indian Major Carps viz.- *Catla catla, Labeo rohita* and *Cirrhinus mrigala* revealing two number of loci for esterase and many more in other isozymes/allozymes in different tissues i.e.- eye and skeletal muscles and observed high incidence of hybridization in hatchery stock. Allelic frequencies of those loci as reported by Simonsen *et al*. (2004) were 0.18, 0.82 in rohu and 1.0 in catla for EST-1 locus with two alleles, 0.63, 0.37 in catla and 0.77, 0.18, 0.05 in rohu for EST-2 locus with three alleles. The present study exhibits presence of four loci (EST-1*, EST-2*, EST-3*, EST-4*, EC: 3.1.1.) in contrast to Simonsen et al. (2004) for catla, rohu as well as all the backcross generations.

In the present study, the dendogram for esterase profile based on genetic distance matrix showed two distinct clusters of carps linked together where one with rohu and excluding catla keeping aside in a separate branch. This indicated strongly that, all the generations of carps are linked more closely to rohu then catla. This isozyme marker proved to be highly significant to reveal segregation pattern in various backcross generations catla and rohu in this study. There exists positive correlation for genetic distance between both the backcross generations and B1R backcrosses were more distantly correlated to rohu then BC_1F_2. Highest value of genetic distance of rohu was observed with catla followed by BC_1F_2, F_2 and B_1R. B_1R backcrosses showed maximum distance with catla and minimum with F_2. The BC_1F_2 generation showed maximum genetic distance with catla followed by rohu, B_1R and F_2.

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Table 1 : Genetic distance correlation (Theta) based on polymorphism of esterase

Theta	Catla	Rohu	F_2	B_1R	BC_1F_2
Catla	***				
Rohu	0.57	***			
F_2	0.42**	0.06**	***		
B_1R	0.51	0.03	0.12	***	
BC_1F_2	0.38	0.02	0.05	0.02	***

*** The values of F_2 are the means of three distinct types of F_2 progenies based on their morphotypes similar to catla, rohu and F_1,*** Minimum correlation of genetic distance (0.00),*

Table 2 : Genetic distance matrix of co-ancestry identity for esterase

	Catla	Rohu	F_2	B_1R	BC_1F_2
Catla	***				
Rohu	0.50	***			
F_2	0.31**	0.07**	***		
B_1R	0.42	0.02	0.12	***	
BC_1F_2	0.25	0.03	0.002	0.02	***

*** The values of F_2 are the means of three distinct types of F_2 progenies based on their morphotypes similar to catla, rohu and F_1, *** Closest genetic distance (0.00)*

Table 3 : Genetic distance values as per Nei (1972)

	Catla	Rohu	F_2	B_1R	BC_1F_2
Catla	***				
Rohu	0.42	***			
F_2	0.21**	0.03**	***		
B_1R	0.29	0.03	0.02	***	
BC_1F_2	0.22	0.05	0.02	0.02	***

****The values of F_2 are the means of three distinct types of F_2 progenies based on their morphometry similar to catla, rohu and F_1, *** Closest genetic distance (0.00)*

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Design and Evaluation of Nanoparticulate Drug Delivery Systems for Imaging and Treatment of Malignant Brain Tumor

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*Abstract-*Malignant brain tumours are one of the most devastating human cancers associated with high mortality and morbidity rates. The median survival of malignant glioma patie-nts ranges between 3 and 16 months and has virtually rema-ined unchanged during the last 3 decades. Difficulties in early detection, local recurrence, and resistance to conventional therapies are the major reasons for failure in malignant brain tumour treatment. The therapy of malignant gliomas is further limited by the inadequate delivery of therapeutic agents to the brain due to the presence of the blood-brain barrier, blood– brain–tumor barrier as well as non-specificity targeting. Nanoparticles (NPs) have drawn increased interest in treating malignant brain tumours due to their potential to act as a vector for brain delivery and to provide tumour-specific detection and treatment. If designed appropriately, NPs may act as a drug vehicle able to target tumor tissues or cells, and protect the drug from inactivation during its transport. The aim of this article was to provide brief overview of nanoparticulate drug delivery systems for imaging and treatment of brain cancer and to evaluate their safety in clinical use. Besides invasive physical methods to bypass or disrupt the BBB and/or BBTB, other methods like pharmacological and physio-logic approach are possible.

Keywords: nanoparticulate, nanoparticle, nanovectors, nanomedicine, nanoparticulate drug delivery system.

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DESIGN AND EVALUATION OFNANOPARTICULATEDRUG DELIVERYSYSTEMS FORIMAGING AND TREATMENTOFMALIGNANTBRAINTUMOR

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Design and Evaluation of Nanoparticulate Drug Delivery Systems for Imaging and Treatment of Malignant Brain Tumor

Minyahil A. Woldu [α], Jimma Likisa Lenjissa [σ] & Gizaw Dabessa Satessa [ρ]

Abstract- Malignant brain tumours are one of the most devastating human cancers associated with high mortality and morbidity rates. The median survival of malignant glioma patients ranges between 3 and 16 months and has virtually remained unchanged during the last 3 decades. Difficulties in early detection, local recurrence, and resistance to conventional therapies are the major reasons for failure in malignant brain tumour treatment. The therapy of malignant gliomas is further limited by the inadequate delivery of therapeutic agents to the brain due to the presence of the blood-brain barrier, blood–brain–tumor barrier as well as non-specificity targeting. Nanoparticles (NPs) have drawn increased interest in treating malignant brain tumours due to their potential to act as a vector for brain delivery and to provide tumour-specific detection and treatment. If designed appropriately, NPs may act as a drug vehicle able to target tumor tissues or cells, and protect the drug from inactivation during its transport. The aim of this article was to provide brief overview of nanoparticulate drug delivery systems for imaging and treatment of brain cancer and to evaluate their safety in clinical use. Besides invasive physical methods to bypass or disrupt the BBB and/or BBTB, other methods like pharmacological and physiologic approach are possible. Different manufacturing methods of nano-formulation have been investigated and these include nano-precipitation, emulsion polymerization, emulsion solvent evaporation, supercritical fluid expansion method, complex coacervation, salting out method, and denaturation. liposomes can serve as a controlled release carrier or simply as a biocompatible solubilizing vehicle for poorly soluble agents. Dendrimers are organic NPs capable of crossing the BBB to deliver therapeutics to tumors. Most inorganic NPs employ an organic polymer as a protective layer so as to increase circulation, half-life and to protect both the particle from the body and the body from the particle. The magnetic NPs can be coupled with diagnostic and therapeutic agents to provide cellular targeting. Quantum dots, Fe3O4 NPs, gold NPs and polymers have all demonstrated levels of success as theranostic NPs. The NPs may be especially helpful for the treatment of the disseminated and very aggressive brain tumors. Unlike biodegradable particles such as liposomes and polymeric-based nanoparticles, metallic NPs are relative newcomers to the field and thus the available toxicology data for each NPs system are limited. In medicine, greater understanding of the origin of diseases on the nanometre is being derived, and drug delivery through function-alised nanostructures may result in improved pharmacokinetic and targeting properties.

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I. Introduction

Cancer is currently the second leading cause of death in Europe, while it shows probably the highest clinical complexity [1]. Malignant brain tumours (gliomas) are one of the most common, aggressive and devastating human cancers associated with high mortality and morbidity rates. Clinical management of these tumours remains challenging despite recent advances in current treatment strategies [2,3]. The median survival of malignant glioma patients ranges between 3 and 16 months and has virtually remained unchanged during the last 3 decades [2].

Difficulties in early detection, local recurrence, and resistance to conventional therapies are the major reasons for failure in malignant brain tumour treatment [2]. Conventional anticancer drugs also exhibit a lack of specificity, poor solubility and distribution, unfavorable pharmacokinetics and high tissue damage or toxicity [4]. The therapy of malignant gliomas is further limited by the inadequate delivery of therapeutic agents to the brain due to the presence of the Blood-Brain Barrier (BBB), blood–brain–tumor barrier (BBTB) as well as non-specificity targeting [2,5,6].

Nano-Particles (NPs) have drawn increased interest in treating malignant brain tumours due to their potential to act as a vector for brain delivery and to provide tumour-specific detection and treatment [2,7]. The development of a drug delivery strategy which can mediate efficient tumor targeting together with high cellular internalization and extensive vascular extravasation is essential and important for glioma treatment [8]. Nanomedicine bears the potential to provide an effective answer to the complexity of the disease as it offers more therapeutic options compared to present conventional therapy [1]. The development of BBB targeting technologies is a very active field of research and development [9]. And therefore, nanoscale technologies are beginning to change the foundations of disease diagnosis, treatment, and prevention [10].

The majority of nanotechnology-based devices useful for cancer therapeutics have been defined as

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nanovectors, which are injectable nanoscale delivery systems [7]. Current progress in nanotechnology and nanomedicine has exploited the possibility of designing tumor-targeted nanocarriers able to deliver radionuclide payloads in a selective manner to improve the efficacy and safety of cancer imaging and therapy [4].

Nanoparticulate technology is of particular use in developing a new generation of more effect-tive cancer therapies capable of overcoming the many boilogical, biophysical, and biomedical barriers that the body stages against a standard intervention [8]. Furthermore, there is a vast array of intriguing nanoscale particulate technologies capable of targeting different cells and extracellular elements in the body to deliver drugs, genetic materials, and diagnostic agents specifically to these locations [10]. Targeted Drug Delivery Systems (DDS) such as passive and active targeting nanocarriers, with diameters ranging from 10-100 nm have been developed to improve the biodistribution, pharmacological, therapeutic and toxicity properties of agents used in cancer diagnostics and therapeutics [4].

Nanoparticles show much promise in cancer therapy by selectively gaining access to tumor due to their small size and modifiability and also due to their ability of taking advantage of fundamental cancer morphology and modes of development such as rapid proliferation of cells, antigen expression, and leaky tumor vasculature. Hence, the application of novel therapeutic agents for the treatment of malignant brain tumours is a timely forwarded request and an urgently needed remedy [8].

The goal of any cancer therapeutics is to preferentially achieve high concentrations of a specific chemotherapeutic agent, a tumor imaging agent, and/or gene therapies at the site(s) of tumors and associated vasculature. Hence, nanovectors must be able to deliver an active agent to achieve effective anti-tumor treatment, or tumor imaging, which is essential for tumor diagnosis and for monitoring the extent and timing of an individual patient's response to anti-tumor therapy [7]. If designed appropriately, NPs may act as a drug vehicle able to target tumor tissues or cells, and protect the drug from inactivation during its transport [11].

The aim of this article is to provide a brief overview of Nanoparticulate Drug Delivery System (NPDDS) for the treatment of malignant brain cancer and to evaluate its safety in clinical usage.

II. Design and Formulation

a) Design

NPs are, in general, colloidal particles, less than 1000 nm, that can be used for better drug delivery and prepared either by encapsulating the drug within a vesicle and or by dispersing the drug molecules within a matrix [12]. The primary consideration when designing any drug delivery system is to achieve more effective therapies by controlling the drug concentration in the therapeutic window, reducing cytotoxic effects, and improving patient compliance [13]. It is also true that effectiveness of the chemotherapy of brain pathologies is often impeded by insufficient drug delivery across the BBB [14] and/or BBTB [6]. The BBB is highly permeable to water, CO_2, oxygen and lipid-soluble substances like alcohol [15]. Many medicines including anticancer drugs are not able to reach the brain due to the lack of drug-specific transport systems through the BBB. Therefore, the development of new strategies based on NPs to enhance the brain drug delivery is of great importance in the therapy and diagnosis of Central Nervous System (CNS) diseases and it is based on the interactions between NPs and the BBB and on their intracellular traffic pathways that the reach of drugs to this system depends. NPs designed to cross the BBB, therefore will be affected by factors like diffusion inside the brain parenchyma, effect of protein corona, BBB alterations in neurological diseases and drug particle size[16].

Small-molecule drugs can be chemically designed or modified (e.g., by prodrug synthesis) to be adequately lipophilic for passive diffusion through the BBB. However, it is important to note that the presence of a brain tumor by itself also disrupts this very selective BBB, and creates an opportunity for the improved delivery of therapeutic agents [17]. Furthermore, drug lipidization results in increased metabolism and peripheral distribution, which necessitates higher doses, potentially at the cost of more frequent adverse reactions. In such cases, or when small drug molecules undergo metabolization in brain endothelial cells, NPDDS formulations should be considered as a means for improving brain delivery [18].

Besides invasive physical methods to bypass or disrupt the BBB and/or BBTB, other methods like pharmacological and physiologic approach are possible. For example, in a number of pharmacological approaches attempt were taken to reduce the relative number of polar groups on the compound of interest in order to enhance BBB and/or BBTB penetration however these approach were ended up with the loss of the activity of the drugs. Similar attempt were also taken to increase the lipophilicity of a molecule, but these trials also ended up with drug susceptible to efflux systems. In general, NPs research takes the advantage of invasive, pharmacological and physiological methods to enhance brain cancer therapy [6].

b) Formulation

Nanostructures and NPs can be used for drug delivery purposes, either as the drug formulation itself or as the drug delivery carrier [19]. Many liposomal, polymer–drug conjugates, and micellar formulations are part of the state of the art in the clinics, and an even

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greater number of nanoparticle (NP) platforms are currently in the preclinical stages of development [13].

The formulation of NPs and physicochemical parameters such as pH, monomer concentration, added stabilizer and ionic strength as well as surface charge, particle size and molecular weight are important for drug delivery [11]. Many different formulations involving NPs have been used for drug delivery purposes, including albumin, poly(D,L-lactic-co-glycolide)acid (PLGA), solid lipid formulations, cetyl alcohol/polysorbate NPs, hydrogels, gold, poly alkyl cyanoacrylate composites, magnetic iron oxide, methoxy poly (ethylene-glycol)/poly(ε-caprolactone), and gelatin [19]. The characteristics of NPs are critically dependent upon the materials used to prepare the NPs [7].

NPDDS are especially important to formulate large-molecule therapeutics potentially efficient to target CNS using peptides, proteins, such as neurotrophic factors, antisense drugs, or genes (plasmids). Owing to their poor stability in biological fluids, rapid enzymatic degradation, unfavorable pharmacokinetic properties, and lack of diffusion towards the CNS [18].

The rapidly advancing field of cancer nanotechnology has generated several innovative DDSs, such as liposomes, dendrimers, quantum dots, iron oxide and carbon nanotubes, to improve and enhance targeted transport of cytotoxic drugs and radionuclides to tumor lesions. In year 2006 only, there were approximately 240 nano-enabled products entered in to the pharmaceutical research pipelines. These nanocarrier systems could provide the delivery platforms needed for improving the delivery of radionuclide to tumor sites [4]. One of the most promising aspects of NP-based cancer therapy is its multifunctionality (Figure 1). NPs can be attached to different types of small molecules such as targeting ligands, imaging, and therapeutic agents to serve as diagnostic and therapeutic agents simultaneously [2].

Different manufacturing methods of nano-formulation have been investigated and these include nano-precipitation, emulsion polymerization, emulsion solvent evaporation, supercritical fluid expansion method, complex co-acervation, salting out method, and denaturation [20].

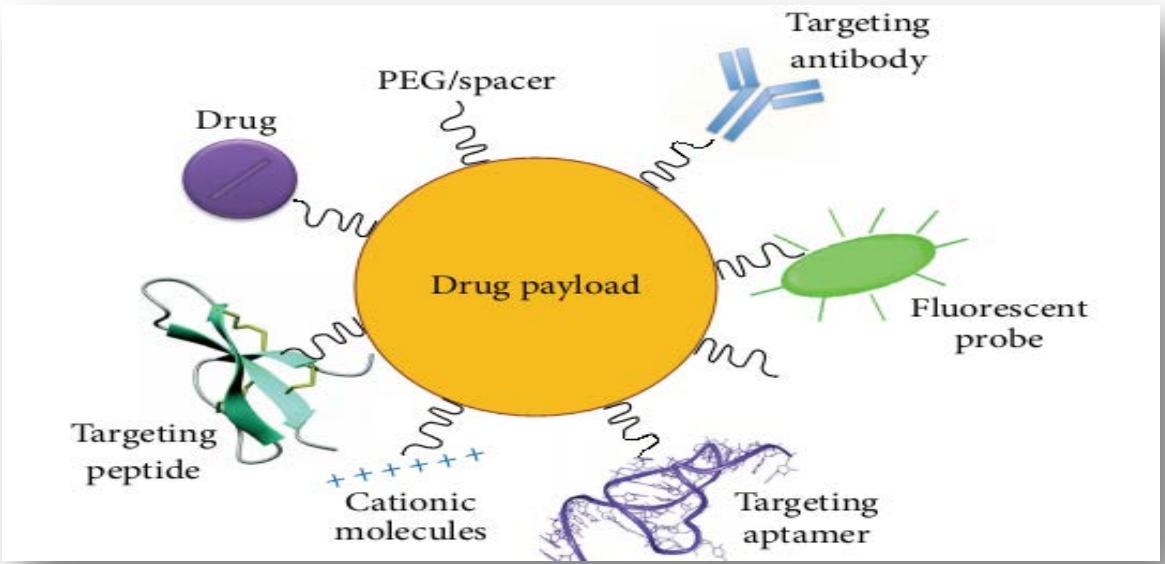

Figure 1 : Multifunctionalized NPs (NPs). A single drug payload can be used as a carrier for multiple molecules with different functionality. Adapted from Reference [16] [16].

III. Lipidic Npdds

a) *Liposomes*

Liposomes, or phospholipid vesicles [21] are type of nanovector made of lipids surrounding a water core. Liposomes are the simplest form of nanovector and their utility is based on the significant difference in endothelial structures-defined as fenestrations-between normal vasculature and tumor-associated vessels[7]. liposomes can serve as a controlled release carrier or simply as a biocompatible solubilizing vehicle for poorly soluble agents [21].

A variety of therapeutic agents have been incorporated into liposomes. Several have reached clinical use. These include liposomal doxorubicin, daunorubicin, amphotericin, cytarabine and verteporfin. Numerous liposomal formulations are in clinical trial, including those for vincristine, all-transretinoic acid, topotecan, and cationic liposome-based therapeutic gene transfer vectors. Many more are in preclinical

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evaluation including liposomal formulations of chemotherapeutics, neutron capture agents, oligonucleotides, plasmid DNA, photosensitizers, antibiotics, and vaccines [22].

The free doxorubicin drug does not cross the BBB. But, is known to bind to DNA-associated enzymes, intercalate with DNA base pairs, and target multiple molecular targets to produce a range of cytotoxic effects by inducing apoptosis and necrosis in healthy tissue while causing toxicity in the brain, liver, kidney and heart. Over the years, many studies have been conducted to devise a drug delivery system that would eliminate these adverse affects of doxorubicin [23].

Study in rats showed that, the employment of poly(butyl cyanoacrylate) NPs result in high efficacy of NPs-bound doxorubicin in intracranial glioblastoma [14]. This result identified that the nano formulations of doxorubicin exhibit favorable pharmacokinetics when compared with the free drug, for example the area under the curve after a dose of 50 mg/m^2 doxorubicin encapsulated in stealth liposomes is 300-fold greater than that of free doxorubicin. Similarly, clearance and volume of distribution can be reduced by at least 250- and 60-fold, respectively by using the nano-formulation [10].

The rate of CNS absorption will also be hastened by using the nano-formulations compared to the free anticancer drugs. For example one study showed that the intravenous injection of doxorubicin with polyfunctional liposomes in rats has shown that the brain of the animals contained the drug already within 15 minutes from injection time [24]. Intravenously injected doxorubicin-loaded polysorbate 80-coated NPs were able to lead to a 40% cure in rats with intracranially transplanted glioblastomas [25,26].

Some key limitations of liposomes include: their propensity to burst release cargo in vivo, a lack of compatibility with various active agents, a limited drug loading volume, the oxidation of liposomal phospholipids, and poor shelf-life stability [27]. "PEGylation," of liposomes using Poly-Ethylene Glycol (PEG) , resulting in "sterically stabilized liposomes" could be helpful for protection against uptake by resident macrophages within the Reticulo-Endothelial System (RES) biobarrier, increasing the circulation time of liposome-encapsulated antitumor agent, resulting in significantly increased therapeutic efficacy [7]. However some studies reported that, the administration of PEGylated liposomes has led to the production of PEG-specific antibodies (immunoglobulin M, IgM), causing the rapid clearance of a further administered dose-leading to an Accelerated Blood Clearance (ABC) phenomena-which further diminishes effective drug concentrations at tumour sites. Fortunately this problem can be rectified by careful tuning of doses [27].

IV. Polymer-based NPDDS

a) *Polymeric NPs*

Polymeric NPs are either nanosized solid particles or capsules which consist of natural or synthetic polymers and to which the drug is attached. They are investigated as drug delivery systems for site-specific targeting of tumours and for the transport of drugs across biological barriers, particularly the BBB [28]. Non-biodegradable polymeric carriers have traditionally been successfully employed in clinically and commercially viable anticancer products. PEG has found a favorite among polymer-protein and polymer-aptamer conjugates, while N-2-Hydroxy-Propyl Methacryl Amide (HPMA) and Poly-Glutamic Acid (PGA) have been used in anti-cancer agents [11,29]. It is generally accepted that for a non biodegradable polymer NP to be able to be excreted it must have a diameter less than the renal filtration cutoff of approximately 5–6 nm [30].

The NPs may offer some advantages such as protection of drugs against degradation, targeting the drugs to specific sites of action, organ or tissues, and delivery of biological molecules such as proteins, peptides, and oligonucleotides [31]. For instance, Poly-Acryl Amide (PAA) nanocapsules, due to their polymeric nature, are stable in biological fluids and during storage, and can entrap various agents in a stable and reproducible way but, since they are not lysed by lysosomal enzymes, their clinical application is restricted [11]. The anticancer drug Abraxane™, the substance paclitaxel stabilised by albumine is one of the drug on the market that uses a (bio)polymeric NPDDS [28]. Nanoparticles may consist of either a polymeric matrix (nanospheres) or of a reservoir system in which an oily or aqueous core is surrounded by a thin polymeric wall (nanocapsules) [11].

The limitation of PNPs include: PNPs do have bioadhesive properties, which may cause them to be immobilized within the mucus or, when in contact with the epithelial cells resulting in a slower clearance from the gastrointestinal tract [11]. There is still very little data available on the long-term fate of polymers and possible toxicity they may generate in neuronal cells [32].

b) *Dendrimers -Based DDS*

Dendrimers are organic NPs capable of crossing the BBB to deliver therapeutics to tumors [33]. Dendrimers are globular macromolecules (5–10 nm) with well-defined tree-like branching architectures and surface functional groups available for further modification [13,28]. Cavities in the core structure and folding of the branches create cages and channels. The surface groups of dendrimers are amenable to modification and can be tailored for specific applications [10]. Dendrimers have remarkable molecular monodispersity and suitable pharmacokinetic properties

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for systemic drug delivery with cleavable chemistry for drug dissociation [13]. Therapeutic and diagnostic agents are usually attached to surface groups on dendrimers by chemical modification [10].

Poly (amidoamine), or PAMAM, is perhaps the most well-known molecule for synthesis of dendrimers. The core of PAMAM is a diamine (commonly ethylenediamine), which is reacted with methyl acrylate and then with another ethylenediamine to make the generation-0 PAMAM. Successive reactions create higher generations of PAMAMs. Functionalization of PAMAMs dendrimers has a dramatic effect on their ability to diffuse in the CNS tissue in vivo and penetrate living neurons following intra-parenchymal or intra-ventricular injections [16].

Dendrimers, like most other organic NPs, exhibit a tunable circulation lifetime and tolerable toxicity [34].

V. Inorganic NPs for Treatment

Studies on Fe_3O_4 NPs have shown success with antibody treatments as well as with thermotherapy induced by an alternating magnetic field. Gold nanoparticles (AuNPs) also offer the ability to achieve noncovalent drug delivery, which allows drugs to be delivered *in vivo* without needing the AuNPs to be taken up into tumor cells. AuNPs can also utilize thermotherapy by heating gold with visible, infrared, or radiofrequency pulses to cause localized tumor damage [6,35,36].

VI. Inorganic NPs for Bioimaging

Most inorganic NPs employ an organic polymer as a protective layer so as to increase circulation, half-life and to protect both the particle from the body and the body from the particle [6,37].

a) *Quantum dots*

Quantum dots (QDs) are nano-scale crystalline structures made from a variety of different compounds, such as cadmium selenide, that can transform the colour of light. QDs absorb white light and then re-emit it a couple of nanoseconds later at a specific wavelength. By varying the size and composition of quantum dots, the emission wavelength can be tuned from blue to near infrared. For example, 2nm quantum dots luminesce bright green, while 5nm quantum dots luminesce red [10]. QDs are the most prolific nanotechnology-based optical contrast agents which are coated with inorganic materials[28].

QDs are useful for studying genes, proteins and drug targets in single cells, tissue specimens, and living animals. QDs are being investigated as chemical sensors, for cancer cell detection, gene expression studies, gene mapping and DNA microarray analysis, immunocytochemical probes, intracellular organelle markers, live cell labeling, medical diagnostics and drug screening [38].

QDs have greater flexibility, when compared to other fluorescent materials, and this makes them suitable for use in building nano-scale computing applications where light is used to process information. These structures offer new capabilities for multicolour optical coding in gene expression studies, high throughput screening, and in vivo imaging [10].

QDs are highly advantageous as they can be tailored for fluorescence emission spectra from 400 to 2000 nm. However, because of their heavy metal content, QDs can potentially be toxic if accumulated in normal tissues without organic polymer protection [39].

b) *Magnetic NPs*

These entities are usually prepared by the alkaline co-precipitation of appropriate ratios of Fe and Fe salts in water in the presence of a suitable hydrophilic polymer such as dextran or polyethylene glycol. These superparamagnetic NPs possess large magnetic moments when brought into a magnetic field, thus producing a localized disturbance in magnetic field homogeneity, but the magnetic memory is lost when the field is removed [Figure 2]. Superparamagnetic NPs can serve as contrast agents in MR imaging to scan tumors, even micro-metastases, as well as in tumor angiogenesis, cell tracking, and gene expression [5,10].

Gadolinium chelates are currently the standard of MRI contrast agents because the gadolinium (III) ion is the best known T_1 contrast agent due to its large magnetic moment [40].

The magnetic NPs can be coupled with diagnostic and therapeutic agents to provide cellular targeting. Stealth NPs can act as pharmaceutical drug delivery devices to penetrate the BBB. A multifunctional NPs polyethyleneglycol-chloro-toxin-fluorophore (NPC-Cy5.5) is capable of targeting glioma cells and is detectable by both MR imaging and fluorescence microscopy [5].

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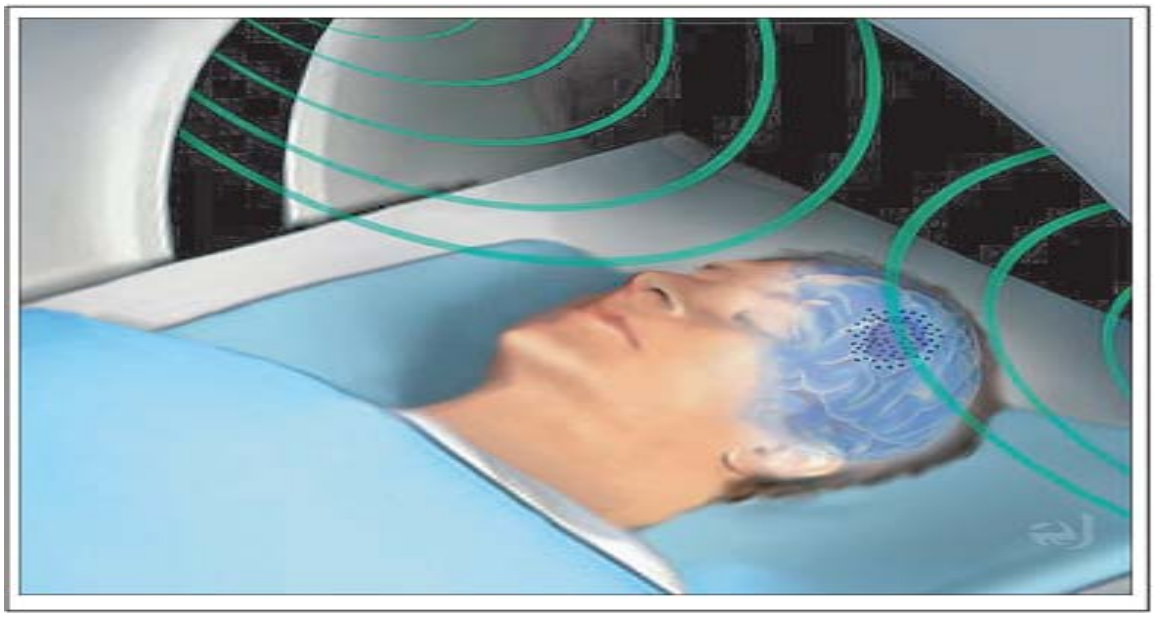

Figure 2 : Intratumoural thermotherapy of a malignant brain tumour with magnetic nanoparticles.

Adapted from Reference [2] [2]. A patient who has undergone intratumoural implantation of magnetic nanoparticles is depicted undergoing an alternating magnetic field session for treatment of his malignant brain tumour by thermotherapy.

To synthesize this nano-probe, iron oxide NPs will be coated covalently with bound bifunctional polyethyleneglycol (PEG) with chlorotoxin, a peptide derived from scorpion venom, and the near-infrared fluorescing molecule Cy5.5. The chlorotoxin peptide binds with high affinity to the membrane-bound matrix metalloproteinase-two endopeptidase, which is preferentially up regulated in gliomas, medulloblastomas, and other tumors of neuroectodermal origin [2,5].

The delivery of the epirubicin-conjugated iron particles was done by intravenous injection of the NPs into a vein, which was located contralateral to the tumor. At the same time, a magnetic field, ranging from 0.5 to 0.8 T, was established and maintained for 45 minutes around the site of the tumor. In both preclinical and clinical studies the magnetic particles were concentrated in the solid tumor and significantly improve the antitumor efficacy of epirubicin treatment [18].

Proteins, including antibodies, can be attached to these magnetic NPs [5]. Two types of iron oxide NPs have been used as imaging agents: superparamagnetic iron oxide (SPIO) and ultra-small superparamagnetic iron oxide (USPIO) NPs [18]. Most MNP formulations are comprised of iron-oxide NPs (IONPs). The major advantage of USPOIs, compared to conventional Gd-(gadolinium-) based contrast agents, is their prolonged MRI contrast effect due to uptake by tumour cells and microglia (reactive phagocytic cells in the brain) and retention within the brain. The route of administration may be oral, parental (subcutaneous, intramuscular, intra-arterial, intravenous) and via the skin [19].

Typically, these NPs are coated with a variety of stabilizing agents including dextran, albumin, starch, or silicones. The major difference between SIPOs and USPIOs relates to their size and circulatory half-life. Both particles may be used as contrasting agents to image the gastrointestinal tract, liver, spleen, and lymph nodes, although the USPIOs may be used to demonstrate blood pooling in diseases such as brain and myocardial ischemia [18].

A sampling of the literature from the past decade finds that magnetic Fe_3O_4 NPs are the most popular inorganic motif for imaging brain tumors [41]. Fe_3O_4 NPs have been shown to be relatively nontoxic with no evidence of tissue damage or pathologic changes in the brain. These particles are also biodegradable [42].

Today, nanocarriers are used in detecting cancer at an early stage, delivering anticancer drugs specifically to malignant cells, and determining if these drugs are killing malignant cells [4]. It has been observed that drug-loaded NPs can have selective distribution to organs/tissues using different types of and proportions of polymers [12]. Inorganic particles require further modification to improve water solubility and stability, with polyethylene glycol (PEG) being popular due to the 'stealth' character during blood circulation and low toxicity [43].

c) Gold Nanoparticles

It makes sense that choosing an inorganic material that is generally inert such as gold than choosing a material that has inherent side-effects such as unchelated gadolinium or QDs [39]. Au NPs can emit

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light so strongly that it is readily possible to observe a single NPs at laser intensities lower than those commonly used for multi-photon absorption-induced luminescence. Au NPs do not blink or burn out, even after hours of observation. These observations suggest that metal NPs are a viable alternative to fluorophores or semiconductor NPs for biological labeling and imaging [18]. Au NPs are able to actively cross the BBB with diameters of up to 50 nm, making them suitable for increased delivery through the disrupted BBTB[44].

Au NPs can be used to enhance contrast of computed tomography imaging [45] [Figure 3]. While inorganic NPs have seen some use in clinical applications for cancer, many still require a greater understanding of their clearance and safety. The inorganic NPs that have seen clinical use are mainly NPs used for MRI contrast agents [6].

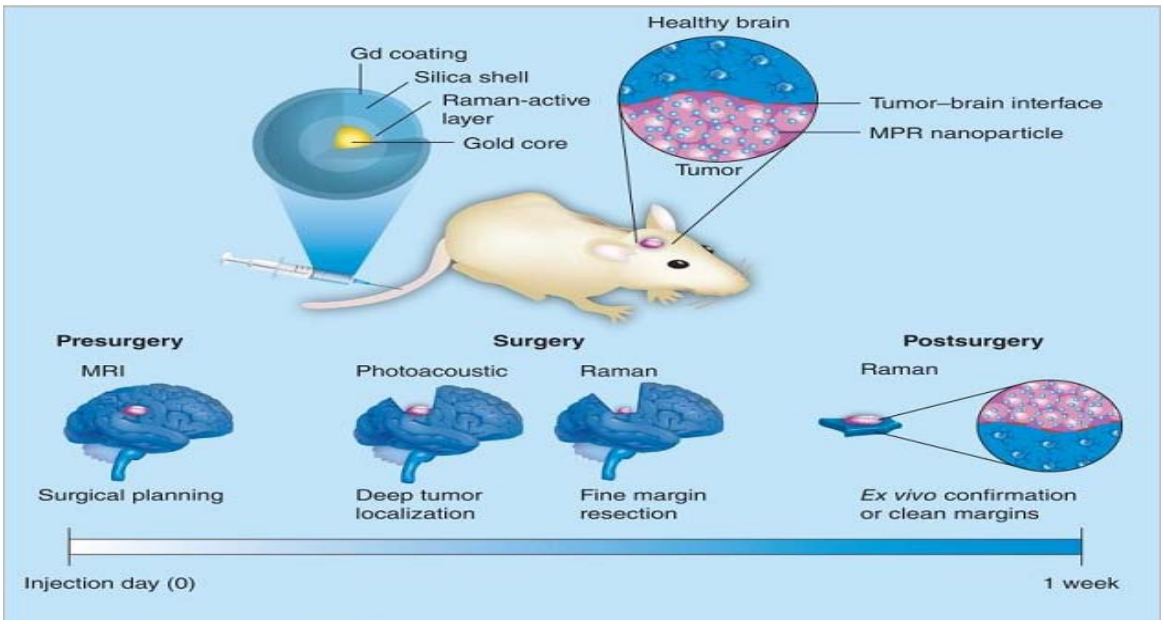

Figure 3 : A gold nanoparticle-based delivery system for identification of brain tumor margins and improved surgical resection. Adapted from Reference [6] [6].
MPR: MRI-photoacoustic-Raman.

VII. Organic NPs for Bioimaging

Liposomes have been investigated for noninvasive real-time monitoring for detection and diagnosis of brain tumors by delivering gadolinium using convection-enhanced delivery. Dendrimers have similarly been used to deliver gadolinium for MRI contrast enhancement as well as fluorescent imaging probes for optical detection of tumors [6].

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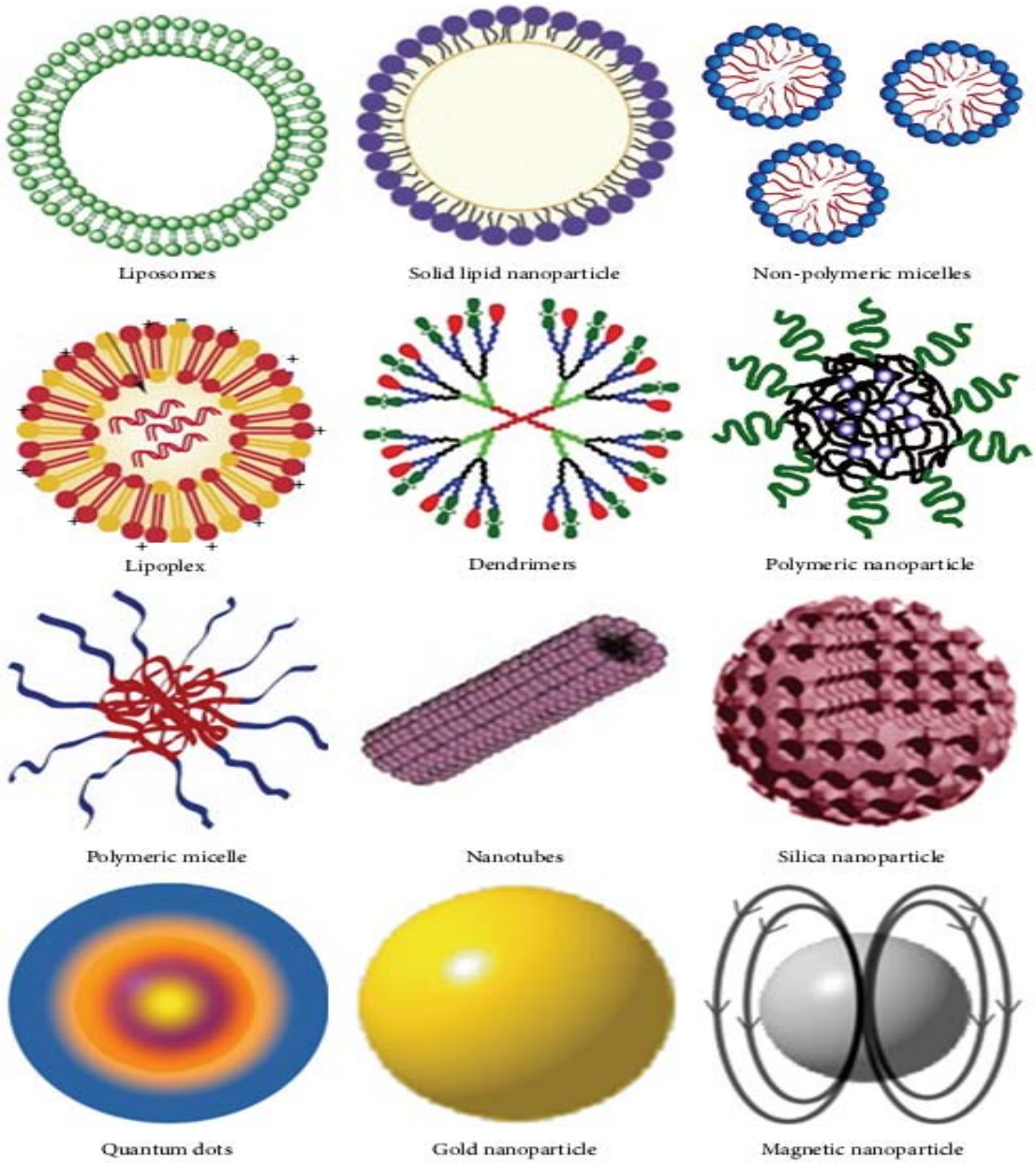

Figure 4 : Different types of NPs. Adapted from reference [16] [16].

VIII. Theranostic NPs

QDs, Fe_3O_4 NPs, Au NPs and polymers have all demonstrated levels of success as theranostic NPs. They have all been used as delivery vehicles to deliver both drugs and imaging agents. However, Fe_3O_4 and AuNPs do have the ability to function as theranostic NPs completely on their own. This is because Fe_3O_4 NPs are magnetic resonance contrast agents, while Au NPs are able to function as computed tomography imaging contrast agents due to their density, and both can be used for thermotherapy [6,46].

IX. Evaluation

a) Merits of NPDDS

The NPs may be especially helpful for the treatment of the disseminated and very aggressive brain tumors [25,26]. Many drugs suffer from rapid breakdown and/or clearance in vivo. Encapsulating the drugs in a protective environment, NPDDSs increase their bioavailability, thereby allowing the clinicians to prescribe lower doses [31]. Nanoparticulate drug delivery system is more effective in delivering of compounds to the brain tumour site in comparison to conventional DDSs [2]. Because, NPs selectively increase the localization of drugs and radionuclides in the tumor through passive targeting or

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active targeting, while sparing non-targeted tissue (the tendency of NPs to accumulate within the brain tumour site via the enhanced permeability and retention effect (EPR)), ensuring minimal drug or radionuclide leakage during circulation, and facilitating intracellular drug or radionuclide delivery and uptake for active targeting [2,4]. This is because the size of the NPs is significantly smaller than a cell; they can deliver a large payload of drugs, contrast agents or fluorescent probe onto the surface or interior of the cell, without disrupting its function [21].

NPs can enter the systemic blood circulation without forming blood platelet aggregates. Their reduced particle size entails high surface area and hence a strategy for faster drug release. Drug delivery rates and particle integrity can be modulated and controlled by engineering carriers in such a way that they can be activated by changes in the environmental pH, chemical stimuli by the application of a rapidly oscillating magnetic field, or by application of an external heat source [21]. As a drug carrier, NPs have significant advantages like better bioavailability, systemic stability, high drug loading, long blood circulation time and selective distribution in the organs/tissues with longer half life [12].

b) Safety and efficacy of nanomedicines

Materials at the nanometer scale often have different physical and biochemical properties from those of the same materials at bulk volume properties that make nanostructures attractive for diagnostic and therapy applications [21]. Reduction in size to the nanoscale level results in an enormous increase of surface to volume ratio, so relatively more molecules of the chemical are present on the surface, thus enhancing the intrinsic toxicity. This may be one of the reasons why NPs are generally more toxic than larger particles of the same insoluble material when compared on a mass dose base [19]. However, data concerning the behaviour and toxicity of particles mainly comes from studies on inhaled NPs [19].

In regards to treatment toxicity, the payload of the NPs can be isolated from the surrounding normal tissues by the addition of biocompatible polymers, preventing the release of the loaded agents within those normal tissues. The result is increased maximum tolerated dose of the therapeutic agent and reduced systemic toxicity. Moreover, targeted delivery of therapeutic agents encapsulated into NPs in conjunction with retention of NPs within the brain tumour site can lead to higher localized concentrations of the agents within the tumour mass, while preventing the undesired systemic consequences of the therapeutic agents [2].

Unlike biodegradable particles such as liposomes and polymeric-based nanoparticles, metallic NPs are relative newcomers to the field and thus the available toxicology data for each NPs system are limited [18]. Several possible mechanisms of action for the toxicity of particles in general have been postulated, including injury of epithelial tissue, inflammation, oxidative stress response, and allergy [19].

Although nanoscale formulation is aimed at enhancing drug delivery without loss of drug activity, a study comparing insulin-chitosan NPs to chitosan solution and chitosan powder formulations showed that the insulin-chitosan NPs were less effective in terms of bioavailability and lowering blood glucose level in both a rat and sheep model [47]. Data from preclinical studies revealed that the particles were cleared by the RES after removal of the magnetic field. Other healthy filtering organs such as the lung and the kidney did not show the presence of the NPs [18].

X. Conclusion and Recommendations

In medicine, greater understanding of the origin of diseases on the nanometre is being derived, and drug delivery through functionalised nanostructures may result in improved pharmacokinetic and targeting properties.

Acronyms

ABC	Accelerated Blood Clearance
Au NPs	Gold Nano-Particles
BBB	Blood-Brain Barrier
BBTB	blood–brain–tumor barrier
CNS	Central Nervous System
DDS	Drug Delivery Systems
HPMA	Hydroxy-Propyl Methacryl Amide
NDDSs	Nano-Drug Delivery Systems
NPs	Nano-Particles
PAA	Poly-Acryl Amide
PEG	Poly-Ethylene Glycol
PGA	Poly-Glutamic Acid
RES	Reticulo Endothelial System

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Competing Interest

The Authors' declare that there are no competing interests.

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Systematic Investigation of Biomass, Fatty Acid Productivity and CO_2 Sequestration from Generator Gases by Fresh Water Microalgae in Photobioreactors for Biodiesel Application

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National Environmental Engineering Research Institute, India

Abstract- The potential of microalgae as renewable energy feedstock for biofuel production is well recognized in developing countries and it is a large source of biomass for capturing CO_2 on non-arable lands. In the current studies photobioreactor with 2 L capacity was used and operated with generator exhaust gases for algal CO_2 sequestration. The reactor operated on generator exhaust gases with CO_2 ranges from 500-8000 ppm. As CO_2 concentration increased from 500 to 4000 ppm, there were steady increase in biomass 450 mg/l of dry weight till 2000 ppm, but above 2000 ppm there is decline in growth. The fatty acid profiles were more or less constant at all the CO_2 concentrations and maximum lipid content was 48%. The maximum reduction in CO_2 was 70% at 500 ppm whereas it was 52% at 4000 ppm. When CO_2 concentration increased to 8000 ppm without air, algae could not sustain and showed decline in biomass content. CO_2 fixation from generator gases not only reduces greenhouse gases but also help in getting fatty acid as biofuel.

Keywords: *CO_2 sequestration, biofuel, fatty acid, algae, generator gases, bench scale laboratory reactor.*

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I. Introduction

The population of the world is increasing day by day; almost billion peoples are added in every 12-13 years. The population of the world was less than 1.6 billion at the beginning of 20th century, currently it is increased to 7.046 billion (PRB, 2014). In addition industries and transportation were increased to provide the needs of this population. As a result of this dramatic change the consumption rate of energy has increased significantly. This creates an uncertainty regarding over-consumption of energy, and also creates a threat of shortage in energy supply, rise in oil price and energy demand. In recent years many countries have initiated energy conservation policy to reduce the over-consumption of energy. However it is projected that the consumption of energy would increase 50 percent in next 20 years (EIA, 2008).

Fossil fuel supplies majority of energy requirement, even though it creates some serious problem of releasing CO_2 to the environment during combustion. The emission of CO_2 to the atmosphere is directly proportional to combustion of fossil fuel (Davis *et al.*, 2011). In last two decades the associated emission of CO_2 from combustion of fossil fuel is increased to 61 % (CDIAC, 2013; EIA, 2013). The continuous emission of CO_2 leads to is accumulated and retain as an extra carbon in the atmosphere, which took million years to mitigate by the oceans and by the earth biosphere.

Concerns about the risks of increasing energy demand, increasing CO_2 emissions and adverse climate changes are prompted to focus on microalgal CO_2 sequestration and biodiesel production. Algal biomass contain high amount of lipid content compared to conventional oil crops, the lipids could be used as a good renewable source of biodiesel. In addition microalgal biomass could be utilized for several applications such as feed for animals, food supplement, nutritional supplement, antioxidants, fertilizers, colorants, immune modulators and natural dyes(Pulz and Gross, 2004; Jhonson and Wen, 2010; Harun et al., 2010). The Omega-3-fatty acids and omega-6-fatty acids play an instrumental role in human health and have large application on medical fields. In particularly DHA is an essential nutrient has an integral role in human neural development, visual development and functioning of central nervous system (Bradbury, 2011).

The present research aimed to sequester CO_2 from generator exhaust gases and fatty acid production by fresh water microalgae in a closed photoreactor. The effect of CO_2 concentration on biomass production and polyunsaturated fatty acid content were investigated.

II. Materials and Methods

a) Algal feedstock

The algal culture was isolated from agricultural runoff grown in Bold's Basal medium (Bischoff and Bold, 1963) Cultures were routinely checked for purity by microscopic examination and plating. consortia of four fresh water microalgal strains were used in this experiment.

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b) Photo bioreactor experiment

CO_2 sequestration studies were performed in a 2L closed borosilicate glass cylindrical photo bioreactor, at room temperature under continuous illumination of fluorescent light. Two fluorescent lamps of 40W were uniformly fitted on the sides of the photo bioreactor. The overall light intensity ranged from 1600 lux to 1800 lux. Fig. 1 showed a schematic diagram of the experimental setup. The air and CO_2 from generator exhaust gas was mixed in a mixing chamber to achieve stable and desired CO_2 concentration in the air stream before entering the photo bioreactor. The feed air stream and outlet air stream were analyzed for CO_2 concentrations. The algal samples were collected from an outlet at regular intervals and analyzed for various parameters.

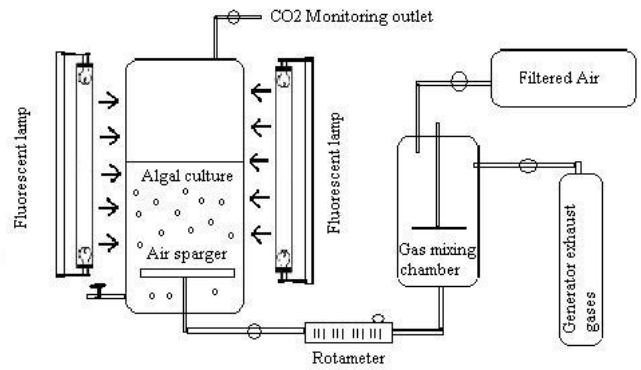

Figure 1 : Schematic diagram of photo bioreactor for sequestering CO_2 from generator exhaust gases by freshwater microalgae

III. Analytical Methods

a) Algal biomass

The concentration of algal biomass was measured by measuring the optical density of the algal suspension at 680 nm (Thermo spectronic, US). The dry weight of algae was estimated from a standard graph. Optical density was calibrated against dry weight measured gravimetrically on pre weighed GF/C glass fiber filters (R^2=0.981)

b) Fatty acid estimation

Algal cells were harvested by centrifugation (10000 rpm) for 10 min. The cell pellets were washed with distilled water and dried. Fifty mg of dried algal biomass was taken in 15 ml of test tube, 1.6 ml of double distilled water, 4 ml methanol and 2 ml of chloroform were added and mixed thoroughly for 30 S. Thereafter, an additional 2 ml of chloroform and 2 ml of double distilled water were added and solution was mixed for 30 S. Following this, the mixture was centrifuged, at 5000 rpm for 10 min. The upper layer decanted and the lower chloroform layer containing the extracted lipids was collected in another test tube. The extraction procedure was repeated again with the residual pellet and both the chloroform extracts were mixed to gather and evaporated till dryness. The dried total lipids were measured gravimetrically and lipid content was calculated as percentage of algal biomass.

Lipids were trans-esterified under nitrogen using BF_3/CH_3OH (12%) for 10 min at 100 °C. Fatty acid methyl esters (FAME) were analyzed using a gas chromatograph (Agilent 7820A, US) equipped with an on-column injector, a DB-Wax (10 m×0.100 mm; 0.10 µm film thickness) capillary column and a flame ionization detector. Fatty acids were identified by comparing their retention times with known standards.

c) Results and Discussion

Since the objective of the study was to evaluate the sequestration of CO_2 from generator exhaust gases by isolated fresh water algae. The CO_2 removal efficiency, growth profile, lipid content and fatty acid profiling were measured at different CO_2 concentrations.

d) Growth of fresh water Algae

The growth profile of the freshwater algae under different CO_2 concentrations from generator exhaust gas is shown in Fig. 2. At all the concentrations, the growth increased steady with time reaching a steady state after about 3 days. The increasing CO_2 concentration from 500 to 4000 ppm enhanced the growth of microalgae, but further increase to 8000 ppm in CO_2 concentration resulted in decreased growth. Hu and Gao (2003) observed enhanced in growth of *Nanochloropsis* sp. when CO_2 concentration was increased from 350 to 2800 ul/l. The maximum specific growth and maximum productivity during CO_2 sequestration was given in Table1. In general, air stream with 500 ppm of CO_2 gave maximum biomass productivity of 54 mg/l with 0.14 g/l specific growth rate. Whereas at 4000 ppm of CO_2, biomass productivity has marginally decreased to 52 mg/l with specific growth rate of 0.16 g/l. Lopes and Franco (2013) have also shown that growth kinetics increased with increased in CO_2 concentration from 5% to 15%, but further increased there is decreased in biomass productivity and specific growth rate. The

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microalgal culture reached the doubling time in 3 days and stationery stage was reached after 10 days for all the concentration of CO_2 Fig. 3 shows that pH of the fresh water microalgal culture was increased continuously in all the concentration of CO_2. The pH during the growth increased from 6.4 to 9.0– 9.5 after eleven days. This may be due to utilization of carbon dioxide which leads to accumulation of free OH- ions. The increasing CO_2 concentration from 500 to 4000 pm, the alkalinity during operation was 470-520 mg/l (Data not shown). This increased in alkalinity levels with the time may be due to formation of bicarbonates. The high alkalinity and alkaline pH are indicative of CO_2 fixation.

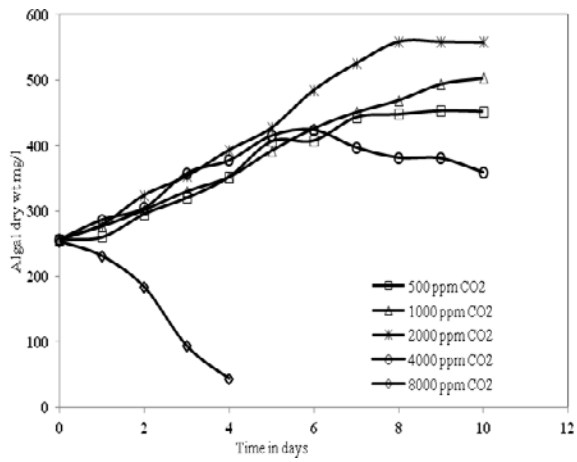

Figure 2 : Growth of fresh water microalgae in photo bioreactor under different concentration of CO_2 from generator exhaust gases

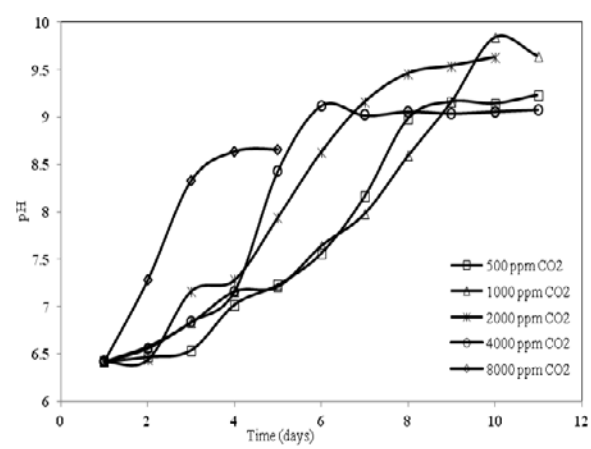

Figure 3 : pH increase of fresh water microalgae in photo bioreactor under different concentration of CO_2 from generator exhaust gases

Table 1 : Maximum biomass and specific growth rate of fresh water micro algae under different concentration of CO_2 from generator gases

CO_2 Concentration of generator gases (PPM)	Maximum biomass productivity mg/l	Maximum specific growth μ
500	54	0.14
1000	40	0.10
2000	58	0.14
4000	52	0.16
8000	-	-

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e) CO_2 removal efficiency of fresh water microalgae

The carbon dioxide removal efficiency of the fresh water microalgae in the photo bioreactor at 500 ppm, 1000 ppm, 2000 ppm, 4000 ppm and 8000 ppm of carbon dioxide are shown in Fig 4. The results of the present study show comparatively higher removal percentage at high CO_2 concentrations. The maximum removal efficiency of fresh water microalgae was 70 %, 63 %, 60 % and 52 % for 500- 4000 ppm of CO_2 respectively. The freshwater algae was observed to be more efficient at higher CO_2 concentration. When the concentration of CO_2 increased to 8000 ppm the maximum removal efficiency declined to 33%. Morais and Costa (2007) reported the similar pattern of CO_2 removal efficiency in cultivating *Spirulina* sp 6 % and 12 % of CO_2, the CO_2 reduction rate was was reduced to 53.29 % to 45.61% at 12 % of CO_2. Keffer and Kleinheinz (2002) was observed CO_2 removal efficiency of Chlorella was reached to a maximum of 74 % at lowere concentration of (1850 ppm) CO_2 than the higher concentration.

According to Lopes et al (2009) longer retention time increases the removal efficiency of the CO_2, it has been reported the removal efficiency increased to 45% by providing longer retention time in sequential reactor. In the present experiment, the retention time has been increased by providing low flow rate. The low flow rate (20 ml/ min) increases the retention time in the photo bioreactor and help the freshwater algae to sequester high amount of CO_2 and decrease the loss of CO_2 from the photo bioreactor.

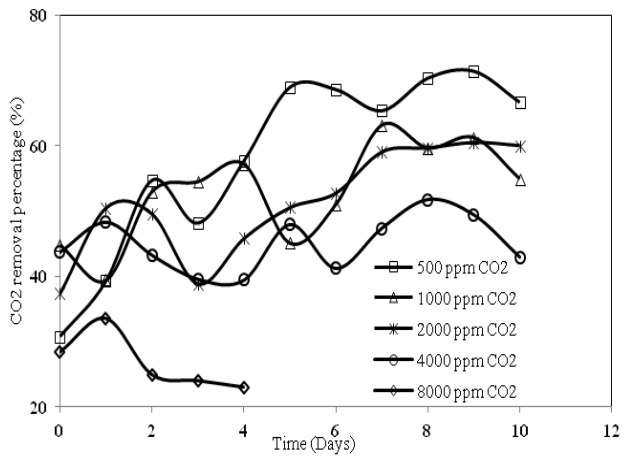

Figure 4 : CO_2 removal efficiency of fresh water microalgae in photobioreactor under different concentration of CO_2 from generator exhausts gases

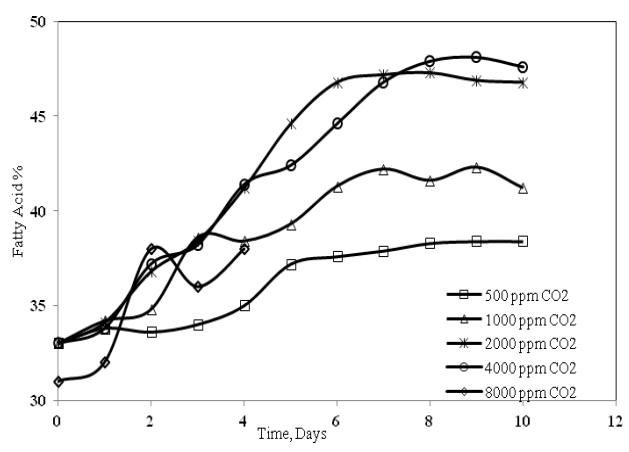

Figure 4 : Fatty acid percentage of fresh water microalgae in photo bioreactor under different concentration of CO_2 from generator exhausts gases

f) Lipid and fatty acid content of fresh water algae

The fresh water alga was not only evaluated for its CO_2 sequestration potential based on its growth, but also for its potential use as a feedstock for biodiesel. This was determined from the fatty acid content of the algal cell given in Fig 5. It was observed that as the cell growth increased with the time according to the concentration of the CO_2 and its fat content was also

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increased in 5 to 10 days. The maximum lipid content was 48.1%, 47.3%, 42.3% and 38.4% for 500 ppm, 1000 ppm, 2000 ppm and 4000 ppm of CO_2 concentration respectively. In general the combinations of high biomass growth accompanied with high lipid content consider as a good lipid producer, however the lipid content and the growth of the microalgae is inversely proportionate (Francisco et al., 2009).

Table 2 : Fatty acid composition of fresh water micro algae under different concentration of CO_2 from generator gases

CO_2 Concentration of generator gases (PPM)	SFA (g/100g of FA)	MUFA (g/100g of FA)	PUFA (g/100g of FA)	TFA (g/100g of FA)
500	56.05	34.27	9.68	<0.01
1000	45.20	36.05	18.75	<0.01
2000	46.08	34.18	19.74	<0.01
4000	49.78	28.85	21.37	<0.01
8000	64.26	26.43	9.31	<0.01

In the present work the results clearly indicate that the fresh water microalgal growth and lipid content was comparatively high in higher concentration of CO_2. However the very high concentration at 8000 ppm of the CO_2 the fresh water microalgae showed declined growth but the lipid content was reached 38%.

The fatty acid profiling was given in Table 2. The polyunsaturated fatty acid proportion was increased when the concentration of CO_2 increased. The PUFA content were increased dramatically from 9.68% to 21.37% with increased in CO_2 concentration from 500 to 4000 ppm. It is noted that the increase in concentration of CO_2 increases the concentration of saturated fatty acids (SFA) and monounsaturated fatty acid (MUFA) content. In all the concentration of CO_2, microalgal lipids were mainly composed of 26-35% MUFA, 9-22 % of PUFA and 45-65% of SFA. The present study demonstrates that elevation of CO_2 concentration could raise the production of PUFA, MUFA, and SFA on dry mass basis. The conversion of CO_2 from generator gas gives "wealth from waste".

IV. Conclusion

Microalgae with fast growing capacity and high lipid content fixes more CO_2 and provide a promising alternative technology for reducing greenhouse gases and fulfil the future energy demand. Our results showed the removal efficiency, fatty acid content under different concentration of CO_2 (500 ppm to 8000 ppm) introduced to freshwater microalgae in a photobioreactor. Algal biomass and lipid productivity was increased in all the concentration, and the removal efficiency CO_2 remained constant in the photo bioreactor. Considering the greenhouse gas emission by the fossil fuel and limited availability, algal biofuels are only the current renewable source for biodiesel.

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Nitrification of Fish Processing Waste Water using Mixed Cultures of *Nitrosomonas* and *Nitrobactor* for Ammonia Degradation (Phase-I)

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Abstract- The present study aims at nitrification of fish processing wastewater using mixed cultures of nitrifying bacteria, Nitrosomonas and Nitrobactor. Parameter optimization of the process is done by using Response Surface Methodology (RSM). Central-Composite design is used to optimize the various parameters for nitrifying cultures. The process parameters namely, the pH, temperature, microbial load and effluent concentration are optimized. Optimized values are obtained from the second order polynomial equation that resulted from the Central-Composite design. The physical - chemical characteristics of fish processing wastewater are presented. Their values show the alkaline nature and high levels of COD and BOD values. The capability of the various models, namely, Logistic, Monod, Herbert, Shehata & Marr, Tessier and Haldane models in representing the batch kinetic data of the present work are reported for mixed nitrifying cultures, while Logistic model is best suited in describing the nitrification of the fish processing waste water. Experimental data collected in a Rotating Biological Contactor using mixed culture, operated on a batch basis for twenty two days with an average organic loading rate of 150 mg/L of ammonia at 20 rpm are reported for every twenty four hours. The future research is proposed for the nitrification of waste water using isolated nitrifying organism from soil for ammonia degradation.

Keywords: nitrification, fish processing waste water, ammonia degradation, nitrosomonas, nitrobactor.

GJSFR-G Classification : FOR Code: 859805p

NITRIFICATION OFFISHPROCESSING WASTE WATERUSING MIXED CULTURESOFNITROSOMONASAND NITROBACTORFORAMMONIADEGRADATION PHASE-I

Strictly as per the compliance and regulations of :

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Nitrification of Fish Processing Waste Water using Mixed Cultures of *Nitrosomonas* and *Nitrobactor* for Ammonia Degradation (Phase-I)

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I. Introduction

Industrial fish processing generates large amounts of waste or residues of high nutrient content which, if not properly utilized or treated, is likely to be deposited in the environment creating pollution and health problems (Hwang and Hansen, 1998; Kotzamanis et al, 2001). Fish processing residues include scales, viscera, fish scrap, fat solids, proteins, fish rejects, and liquid stick waste water (UN Report, 1997; Hwang and Hansan, 1998).

The solid fish make up 30-40% of the total production, depending on the species processed. The waste water from seafood processing plant contains large amounts of organic matter, small particles of flesh, breading, soluble proteins and carbohydrates. Mauldin and Szabo (1974) reported that as much as 65% of the tuna is wasted in the canning process. The average daily waste flow is over 27,000 Lits ton^{-1} of fish, varying from 500 – 1550 mg Lit^{-1} of BOD; 1300 – 3250 mg Lit^{-1} of COD; and 17,000 mg Lit^{-1} of TSS of which 40% was organic. Steven (1981) surveyed the North Carolina seafood industry and stated that unloading, washing and separating ice, sorting grading and re-icing before shipping all constitute handling and sources of contamination form personnel. Results of the survey found that the average values for TSS was 2.4, ash 1.11, organic solids 1.30 and BOD 0.23 g kg^{-1} while water use was 1.51kg^{-1} of fish handled.

a) *Characterization of fish processing waste water*

The volume and concentration of wastewater from fish processing depends mainly on the raw fish composition, additives used, processing water source and the unit process. The main components of fish processing wastewater are lipids and protein (Gonzalez, 1996).

i. *pH*

Effluent pH from fish processing plants is usually close to neutral. The results obtained from four different fisheries from British Columbia showed pH in the range of 5.7 – 7.4 with an average pH of 6.48 (Technical Report Series FREMP, 1994).

ii. *Solid Content*

Fish processing wastewater contains high levels of suspended solids which are mainly proteins and lipids (Palenzuela-Rollon et al., 2002). The fish condensate has high volatile solids (VS) consisting of trimethyl amine (TMA) and volatile fatty acids (VFA). The wastewater characteristics from fish processing units depend on the composition of raw fish, the unit processes, source of processing water and additives used such as brine, oil for the caning process (Palenzuela – Rollon, 1999).

iii. *Fat, Oil and Grease (FOG)*

Fat, oil and grease (FOG) are also important parameters of fish processing wastewater. Around 60% of the oil and grease originates from the butchering process (Nova Tec, 1994).

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b) Environmental problems

The main environmental problems linked to seafood cannery industries are wastewater, emissions to the atmosphere, solid wastes (with similar characteristics to urban wastes), inert and hazardous wastes (IHOBE 1999). The main atmospheric emissions are due to the use of fuel to generate steam. In addition, there are other emissions in the form of odors, because of the presence of hydrogen sulphide and trimethylamine (Xunta de Galicia 2001).

Wastewaters generated in seafood processing factories have quite a high organic load (mainly from proteins and fats) and also high levels of salinity. These effluents exert a strong impact on the environment, being especially relevant when they are discharged into seawater with low self-purification capacity, as occurs in the estuaries of Galicia, where the largest number and highest density of these factories in Europe are located. Fish and shellfish canning industries in Galicia and also in Spain generate different liquid effluent that present a wide range of characteristics according to the raw material processed (tuna, mussel, sardine, mackerel, etc). Cooking effluents contain the highest organic matter load. The high salinity (Na^+, Cl^-, SO_4^{2-}) is caused both by the raw material (fish and shellfish) and the seawater used in the process.

c) Biological treatment processes

After suitable primary treatment the wastewater is treated through a biological wastewater treatment system where microorganisms are involved in degradation of organic matter. Biological treatment processes carried out in two ways:

Aerobic processes - biological treatment processes that occur in the presence of oxygen.

Anaerobic processes – biological treatment processes that occur in the absence of oxygen.

Anaerobic treatment converts the organic pollutants (COD, BOD_5) in wastewater into a small amount of sludge and a large amount of biogas (methane and carbon dioxide), while leaving some pollution unresolved. The main advantages, particularly for bigger plants, are i) low operating costs, ii) low space requirements, iii) valuable biogas production, and iv) low sludge production. Anaerobic systems are well suited to the treatment of fish processing wastewater because a high degree of BOD_5 removal can be achieved at a significantly lower cost than comparable aerobic systems and generate a smaller quantity of highly stabilized, and more easily dewatered, sludge. Furthermore, the methane – rich gas which is generated can be captured for use as a fuel (Johns, 1995).

d) Nitrification

Nitrification is the aerobic oxidation of ammonium to nitrate. It consists of two sequential steps carried out by two phylogenetically unrelated groups of aerobic chemolithoautrophic bacteria. Some heterotrophic bacteria can also oxidize ammonium to nitrate, but this is only a very small contribution to the overall ammonia oxidation. First, ammonium is oxidized to nitrite by the aerobic ammonia – oxidizing bacteria. Approximately 2moles of protons are produced for every mole of ammonium oxidized. Ammonium oxidization is therefore an acidifying reaction. In the second step nitrite is oxidized to nitrate by the nitrite oxidizing bacteria. No single known autotrophic bacterium is capable of complete oxidation of ammonium to nitrate in a single step. The key reactions of nitrification are given by,

$$NH_4^+ + \tfrac{3}{4}\, O_2 \rightarrow NO_2^- + H_2O + 2H^+ NO_2^- + \tfrac{1}{2}\, O_2 \rightarrow NO_3^-$$

The main objective of this study was to degrade the ammonia present in the fish processing waste water. The fish processing waste water was analyzed for total solids, dissolved oxygen, nitrite, nitrate, ammonia, BOD and COD. Microbial consortium of nitrifying bacteria was obtained from culture collection centre. The process parameters for the nitrification were optimized and using the optimized conditions a Rotating Biological Contactor (RBC) was operated.

II. Materials and Methods

a) Chemicals used

The chemicals used in this study are of the best quality and the analytical grades. Distilled water is used throughout the experimental work.

b) Experimental methods

i. *Microbial cultures and maintenance*

The nitrifying strains of *Nitrosomonas and Nitrobactor* were obtained from National Collection of Industrial Microorganism Laboratory, Pune, India. The growth medium for the strains was prepared by mixing 100ml of solution-I and 5ml of solution-II. The composition of solution-I consists of: $MgSO_4.7H_2O$-0.2 gL^{-1}; K_2HPO_4-1.0gL^{-1}; $FeSO_4.7H_2O$-0.05gL^{-1}; $CaCl_2.4H_2O$-0.02gL^{-1}; $MnCl_2.4H_2O$-0.002gL^{-1}; and $Na_2MoO_4.2H_2O$-0.001gL^{-1}. The pH of the solution was then adjusted to 8.5 using 1N Na OH. After adjusting the pH, 0.5% of $CaCO_3$ was added to solution-I used for the growth of *Nitrosomonas*. Solution-II was prepared by dissolving 6g of $NaNO_2$ and 3g of NH_4Cl in 1L of distilled water for *Nitrobactor* and *Nitrosomonas* respectively. 100ml of solution-I was transferred in to each Erlenmeyer flask, which was cotton plugged and then sterilized in an autoclave at 121°C for 20 min. After cooling, 5ml of filter sterilized solution-II was added aseptically to reach a final concentration of 150mg. The media was then inoculated with 1ml of the corresponding culture and the flasks were placed in the incubator shaker at 30°C for 5 days. The culture was stored at 4°C in refrigerator.

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The fish processing wastewater samples investigated in the study were collected from the local fish market. The samples were transported in cool boxes to the laboratory for analysis.

ii. *Rotating Perforated Tubes Bio film Reactor*

Rotating perforated tubes bio film reactor is a novel bioreactor employed in this study for treatment of fish processing wastewater. Rotating biological contactors is a remediation technology used in the secondary treatment of fish processing wastewater. This technology involves allowing wastewater to come in contact with a biological medium in order to facilitate the removal of contaminants.

A rotating biological contactor (RBC) consists of discs mounted on a shaft which is driven so that the discs rotate at right angles to the flow of settled sewage. The discs are usually made of plastic (polythene, PVC, expanded polystyrene) and are contained in a trough so that about 40 percent of their area is immersed. The discs are arranged in groups or packs with baffles between each group to minimize surging or short circuiting. With small units the trough is covered and large units are often housed within buildings. This is reducing the effect of weather on the active bio film which becomes attached to the disc surfaces.

iii. *Advantages of Rotating Biological Contactors (RBC)*

- Short contact periods are required because of the large active surface.
- They are capable of handling a wide range of flows.
- Sloughed biomass generally has good settling characteristics and can be easily separated from waste stream.
- Operating costs are low because little skill is required in plant operation.
- Short hydraulic retention time.
- Low power requirements.
- Low sludge production
- High efficiency & easy to operate

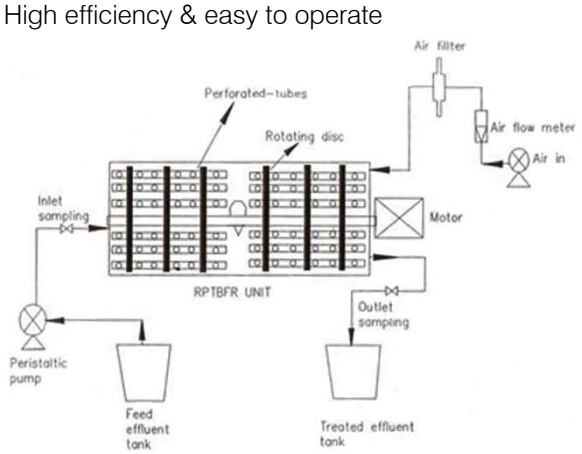

Figure 1 : Schematic diagram of rotating perforated tubes biofilm reactor

c) *Assay methods*

i. *Estimation of suspended solids*

100 ml of the given water sample was taken and filtered using pre weighed what man No.1 filter paper. After filtration, the filter paper along with the suspended solids was dried at 110°C in an oven and the dry weight was taken. The amount of suspended matter present in 100 ml of the given sample was obtained as the difference in weight of the filter paper and converts the value into pm.

ii. *Determination of total dissolved solids*

50 ml of the filtered water sample was taken in a pre weighed china dish. The water content in the china dish was completely evaporated by keeping in an oven at 110°C. After evaporation, the china dish was cooled and weighed. The difference in weight of the china dish was taken as the amount of total dissolved solids present in 50 ml of the filtered water sample and the value was represented in pm.

iii. *Estimation of dissolved oxygen (DO)*

The dissolved oxygen in the water sample was analyzed using Winkler titration method. A series of reagents namely manganous sulphate, concentrated sulphuric acid and alkaline iodine were added in to the sample contained in an air tight BOD bottle. Manganous sulphate when react with potassium hydroxide forms manganese hydroxide which then oxidize to manganic hydroxide by dissolved oxygen in the sample. Manganese hydroxide then converts to manganese sulphate on reaction with sulphuric acid and this in turn liberate free iodine when reacted with potassium iodide. The amount of iodine liberated is obtained by Titrating the above reaction mixture against standard sodium tio sulphate and this represents the amount of dissolved oxygen in the sample. 2.5ml of sample was diluted to 250ml using distilled water and the diluted sample was taken in a 250ml BOD incubator. 2ml of manganese sulphate was added followed by 2ml of alkaline iodine reagent immediately after collection. The tip of the pipette should be below the liquid level while adding these reagents and close with the stopper immediately. Mix well by inverting the bottle 2-3 times and allow the precipitate to settle. Then add 2ml concentrated sulphuric acid. Mix well till precipitate goes into solution. Transfer 50ml sample into a clean conical flask and titrate against standard sodium thio sulp hate until a very pale straw yellow color remains. Add 1ml of starch indicator (blue color develops). Disappearance of blue color is the end point.

iv. *Estimation of nitrite*

The nitrite content in the water sample was analyzed using a colorimetric method. In acid conditions, nitrite forms a diazo-compound with sulfanilamide. This diazo-compound reacts with N-(1-naphthyl)-ethylenediamine (NED) forming an intensely colored pink azo dye, the intensity of which is related to

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the nitrite concentration and can be measured photometrically. Stock solution of nitrite was prepared by dissolving 1.5g sodium nitrate in 1000ml of distilled water. 10ml of the stock nitrite solution was diluted to 100ml with distilled water to obtain the working standard. From the working standard, 2, 4, 6, 8 and 10 ml was pipette out in to a series of volumetric flasks and made up to 100ml using distilled water. 40ml of each dilution was taken in a 50ml volumetric flask and 2ml of sulfanilamide solution was added and thoroughly mixed. After 3-6 minutes 2 ml of NED solution was added and diluted up to 50 ml and mixed well. The color developed during the reaction was read at 543 nm. The calibration chart was prepared by plotting absorbance value verses the corresponding concentrations. 40ml of filtered sample was taken in a 50ml volumetric flask and the same procedure was repeated to read the absorbance.

v. *Estimation of nitrate*

The nitrate content in the water sample is analyzed by a spectrophotometric method. The determination is based on the reduction of nitrate to nitrite in the presence of Zn/NaCl. The produced nitrite is subsequently diazotized with sulfanilamide then coupled with NED to form an azo dye which is measured at 543 nm. Stock solution of nitrate was prepared by dissolving 1.3g of sodium nitrate in 1L of distilled water. Pipetted out 10 mL of nitrate stock solution to a beaker, added 5 mL of concentrated HCl and 2mL of Zn/Na Cl granular mixture and was allowed to stand for 30 minutes, with occasional stirring to form nitrite. The solution was then filtered using what man filter paper and made up to 100ml using distilled water to obtain the working standard. 2, 4, 6, 8 and 10ml were pipette out and made up to 100ml using distilled water. The reduced nitrate was obtained by analyzing the nitrite liberated using the previous described method. Calibration chart was obtained by plotting absorbance verses corresponding nitrate concentration. 10ml of the filtered sample was taken in a beaker and the same procedure was followed to read the absorbance. The nitrate content was determined from the calibration chart.

vi. *Determination of ammonia*

Stock solution was prepared by dissolving 3.141g of ammonium chloride in 1000ml of distilled water. Working standard was prepared by diluting 10ml of the stock solution into 100ml using distilled water. 2, 4, 6, 8 and 10ml of the working standard were pipetted out and made up to 100ml. To 100ml of each dilution 1 mL of $ZnSO_4$ solution was added and mixed thoroughly. The pH of the sample was adjusted to 10.5 using 6N Na OH and allowed to stand for a few minutes and filtered. Initial 25ml was discarded and the rest was collected. 50 mL of the aliquot was taken in a Nessler tube and 0.05 mL EDTA solution or 0.1 mL Rochelle salt was added and mixed well. To this 2 mL of Nessler reagent was add and mixed well by capping Nessler tubes and allowed to stand for a few minutes. The absorbance was measured at420 nm using 1cm cuvette with a reagent blank. Absorbance was plotted against the concentration of ammonia to obtain the calibration chart. 100ml of filtered sample was taken and the same procedure was followed to read the absorbance at 420pm. The concentration of ammonia in the sample was directly derived from the calibration chart.

vii. *Determination of Biological Oxygen Demand (BOD)*

Biological oxygen demand is the amount of oxygen required by the mixed microbial population for the biological decomposition of the organic matter in water under aerobic conditions.

The method consists of filling with sample, an airtight bottle of 250ml and incubating it at 20°C for 5 days. Dissolved oxygen (DO) is measured initially and after incubation, and the BOD is computed from the difference between initial and final dissolved oxygen. Because the initial DO is determined shortly after the dilution is made, all oxygen uptake occurring after this measurement is included in the final BOD measurement. The dissolved oxygen is determined by Winkler method. 2.5ml of the effluent was diluted to 250ml using dilution water in two separate volumetric flasks. One bottle was kept a BOD incubator and incubates at 20°C for 5 day for the determination of final DO content. To the other BOD bottle, 2ml of manganese sulphate was added followed by 2ml of alkaline iodine reagent immediately after collection. The tip of the pipette should be below the liquid level while adding these reagents and close with the stopper immediately. The contents in the bottle were mixed well and the precipitate was allowed to settle. Then add 2ml concentrated sulphuric acid. Mix well until the precipitate goes into solution. Transfer 50ml sample into a clean conical flask and titrate against standard sodium thiosulphate until a very pale straw yellow color remains. 1ml of starch indicator was added which develops a blue color. Disappearance of blue color is the end point. Determine the DO in the sample on the initial day and 5th day. BOD_5 was calculated as follows:

$$BOD_5 = \frac{D1-D2}{P}$$

Where, D1-DO of diluted sample immediately after preparation (mg/L); D2-DO of diluted sample after 5 day incubation (mg/L) and P, decimal volumetric fraction of sample used.

viii. *Determination of Chemical Oxygen Demand (COD)*

Chemical oxygen demand (COD) represents the amount of oxygen required to oxidize all the organic and inorganic pollutants present in the effluent. The organic matter present in the effluent was oxidized by potassium dichromate in the presence of sulphuric acid

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to carbon dioxide and water. The excess potassium dichromate remaining after the reaction is titrated with ferrous ammonium sulphate. The volume of dichromate consumed gives the oxygen required for the oxidation of organic matter. Known quantity of wastewater sample was pipetted out into a 500ml standard flask. (The sample, if highly polluted, can be suitably diluted to a known quantity and an aliquot sample is taken). 20ml of standard dichromate solution was pipetted out into the same distilling flask. With gentle mixing 20ml of concentrated sulphuric acid was added carefully. 0.2 g silver sulphate was added followed by 20ml water. Put one or two glass beads, and then attach the reflux condenser. The solution was refluxed for 2hrs over a heating mantle or Bunsen flame. After cooling, the inside of the condenser was rinsed with small amount of distilled water and collect the washings into the distilling flask.

The excess dichromate present in the solution was titrated against standard ferrous ammonium sulphate, using 0.5ml of ferro in indicator. The end point is blue to red. A blank was also performed with distilled water instead of sample of wastewater and the same procedure was followed. The COD of the sample was calculated as follows:

$$\text{COD}=\frac{(V1-V2)\times N\times 1000}{X}$$

Where, V1-the volume of titrate value with blank; V2-the volume of titrate value with sample; N-the normality of ferrous ammonium sulphate and X-the volume of water sample.

d) Optimization of parameters

i. *Response surface methodology (RSM) using Central Composite Design (CCD)*

RSM is a very effective and most popular statistical tool to optimize the variables having equal importance and influence on each other in the ammonia degradation. The effect of significant process parameters such as pH, temperature, microbial load and effluent concentration were tested for their significance on ammonia degradation.

ii. *Statistical analysis and modeling*

The data obtained from RSM on ammonia degradation are subjected to the analysis of variance (ANOVA). The experimental results of RSM are fitted through the response surface regression procedure using the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j$$

in which Y, the predicted response; X_iX_j, independent variables; β_0, offset term; β_i, ith linear coefficient; βii, i^{th} quadratic coefficient and β_{ij}, the ij^{th} interaction coefficient. However, in this study, the independent variables are coded as X_1, X_2 and X_3.

The chosen independent variable (X_i) levels are coded as x_i according to the equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, i = 1,2,3,\ldots\ldots, k,$$

Where x_i, the dimensionless value of an independent variable; X_i, real value of an independent variable; X_0, real value of the independent variable at the center point and ΔX_i, the step change value.

Thus, the second order polynomial model equation for four factors takes the following form:

$$Y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_4 + \beta_{11}x_1^2 + \beta_{22}x_2^2 + \beta_{33}x_3^2 + \beta_{44}x_4^2 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{14}x_1x_4 + \beta_{23}x_2x_3 + \beta_{24}x_2x_4 + \beta_{34}x_3x_4$$

The Central-Composite design was adopted in order to optimize the important process parameters namely; pH of the medium, temperature, microbial load and effluent concentration for both mixed and isolated nitrifying culture. A five level design (-2, -1, 0, 1, 2) with seven central points was used for the study. The coded and the actual values of the parameters used in the design are presented in Table 1. The design allow to vary the factors simultaneously and to give the individual, interactive and square effects of the parameters associated with the ammonia degradation through a second order polynomial equation.

The statistical software package, MINITAB version 15.0 was used for the regression analysis of the experimental data, and also to plot the contour and response surface plots. The statistical significance of the model equation and the model terms were evaluated via the Fisher's test. The Fisher's variance ratio, F value = (Sr^2/Se^2), is the ratio of the mean square owing to regression to the mean square owing to the error. It is a measure of the variation in the data about the mean. The significance of each coefficient was determined using Student's *t*-test.

Table 1: The coded and actual values of the variables used in the design

Parameters	Coded values				
	-2	-1	0	+1	+2
pH- (A)	7	7.5	8	8.5	9
Temperature (^{0}C) – (B)	20	25	30	35	40
Microbial load (% v/v) (C)	1	2	3	4	5
Effluent concentration (%)-(D)	20	40	60	80	100

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The quality of fit of the second-order polynomial model equation is expressed using the coefficient of determination (R^2) and the adjusted R^2. Coefficient of determination, R^2, is defined as the ratio of the explained variation to the total variation and is a measure of the degree of fit. It is also the proportion of the variability in the response variables, which is accounted for by the regression analysis. When R^2 approaches unity, the better the empirical model fits the actual data. The smaller the value of R^2, the less relevance of the dependent variables in the model has in explaining the behavior variation.

The fitted polynomial equation was then expressed in the form of contour plots and also three-dimensional surface plots, in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study. The point optimization method was employed in order to optimize the level of each variable for maximum response. The combination of different optimized variables, which yielded the maximum response, was determined in an attempt to verify the validity of the model.

e) Growth kinetics

In general, the Monod model has been used for representing the microbial fermentation kinetics. The Monod model which includes the substrate concentration limits the applicability of the model for most of the cases. Instead, the logistic model which relates the growth rate with the initial and final cell mass concentrations could be used to predict the growth behavior of many strains.

i. *Logistic model*

The logistic model states that the rate of growth of the cell is proportional to the cell mass concentration present at any time. When the cell mass reaches the stationary phase there is no growth and hence the rate becomes zero. The growth rate thus depends on how far the cell mass concentration is away from the stationary phase.

$$\frac{dX}{dt} = kX(X_S - X)$$

Where, k is a constant defined as the specific growth rate. The above Equation implies that the growth rate increases with an increase in cell mass concentration and is independent of the substrate concentration.

ii. *Substrate Utilization Kinetics*

Substrate consumption depends on the magnitude of three sink terms, the instantaneous cell mass growth rate, the instantaneous product formation rate and a cell mass maintenance function. The assumed kinetic form is a linear combination of these terms (Weiss and Oll is, 1980) and is given by,

$$\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt}$$

This equation is used for substrate utilization kinetics. The yield coefficient of biomass ($Y_{X/S}$) is based on the substrate utilized which is defined as follows:

$$Y_{X/S} = \frac{Cell\ mass\ produced}{Substrate\ consumed} = -\frac{\Delta X}{\Delta S} = \frac{X - X_0}{S_0 - S}$$

These equations are rearranged and integrated with the set of initial conditions $X=X_0$ and $S= S_0$ gives,

$$S = S_0 - \left[\frac{X - X_0}{Y_{X/S}}\right]$$

III. Results and Discussion

a) Physical-chemical characteristics of fish processing waste water

Table 2 shows the results of physical-chemical characteristics of fishery waste water. The COD (250 mg/L) and BOD (20 mg/L) values were above the permissible limits and are due to the presence of high inorganic and organic matters (Gonzalez et al, 1996). Effluent has high ammonia concentration; therefore the pH value is high. The ammonia concentration mainly depends upon the blood and slime content in the fishery waste water. The high TSS is due to high water-insoluble fats and lipids present in the wastewater (Palenzuela – Rollon, 1999). These results have been observed from the calibration chart which is discussed previously.

Table 2 : physical-chemical characteristics of fish processing waste water

Parameter	Measured value
pH	7.22
Colour	Dirty brown
Total ammonia	250 mg/L
Total nitrite	17.6 mg/L
Total nitrate	6.25 mg/L
TSS	4000 mg/L
TDS	1100 mg/L
BOD	9003 mg/L
COD	11040 mg/L
DO	41.04 l/L

b) Process parameter optimization using central composite design for ammonia degradation using mixed culture

The design matrix which consists of 31 experimental runs was constructed, in order to arrive at a second order polynomial equation to predict the ammonia degradation system. The design matrix and their corresponding experimental and the predicted values are given in Table 3 after the reference. The experimental results suggest that the maximum values of ammonia degradation were obtained for the runs with the central points. The experimental runs of 4, 12, 15,

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27, 29, 30, and 31 produced the highest ammonia degradation (129 mg/l). The results were analyzed using the analysis of variance (ANOVA) and the estimated coefficients are presented in Table 4 after the reference section.

ANOVA results of the data indicate that the model terms, A, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2 are significant ($P < 0.05$). The model term B only insignificant. The need for model reduction to improve the model does not arise because all the terms of the model are significant except B. Thus, it is clear that the linear, square and interactive effects of pH, microbial load, and effluent concentration are significant. Temperature is insignificant. The model F-value was 849.31. The high F-value and non-significant lack of fit indicate that the model is a good fit. The P-values for the model (<0.0001) from the analysis also suggested that the obtained experimental data was in good fit with the model. The regression equation coefficients were calculated and the data was fitted to a second order polynomial equation. The ammonia degradation using a mixed culture of *Nitrosomonas* and *Nitrobactor* can be expressed in terms of the following regression equation:

$$Y = 129-6.83A+0.83B-2.75C+12.08D-5.63AB-3.63AC-13.38AD-1.37BC-7.63\,BD+4.88CD-23.06A^2-27.31B^2-10.94C^2-22.69D^2$$

where, A: pH; B: temperature; C: Microbial Load; and D: Effluent Concentration.

The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.9987 (a value > 0.1 indicates the fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model is capable of explaining 99.87% of the variation in response. This is supported by the parity plot between the experimental and predicted ammonia degradation using mixed culture, as illustrated in Figure 2. The 'adjusted R^2' (0.9976) and the 'predicted R^2' (0.9927) are in reasonable agreement, which indicates that the model is good. The 'adequate precision value' of the present model was 83.687, and this also suggests that the model can be used to navigate the design space. The 'adequate precision value' is an index of the signal-to-noise ratio, and values of higher than 4 are essential prerequisites for a model to be a good fit.

Three dimensional surface plots are drawn to determine the optimum values of the four parameters and are shown in Figure 3.1 to 3.6. The three dimensional surface plot shown in Figure 3.1 explains the interactive effect of pH and temperature on the degradation of ammonia. The maximum value of ammonia degradation lies between 7.9 to 8.1 and 29°C to 31°C.

The Figure 3.2 depicts the interaction between pH and microbial load. The trend observed that maximum degradation of ammonia is obtained at the central point of microbial load and pH for it lies between 2.5 (v/v) to 3 (v/v) and 7.9 to 8.1 respectively.

The Figure 3.3 explains the interactive effect of pH and effluent concentration on the degradation of ammonia. The maximum value of ammonia degradation lies between the pH and effluent concentration range of 7.9 to 8.1 and 64 % to 72 % respectively.

Figures 3.4, 3.5 and 3.6 also showed similar trends as that of previous one. The following are the optimum values obatained by solving the second degree polynomial equation:

pH: 8; temperature: 30° C; microbial load: 3 %(v/v) and effluent concentration: 60%. These optimum values were maintained for all further studies.

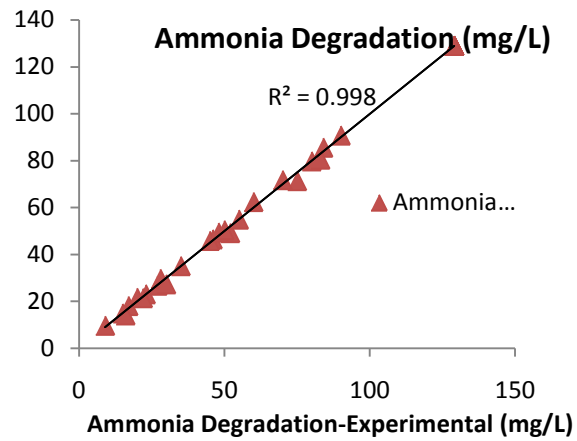
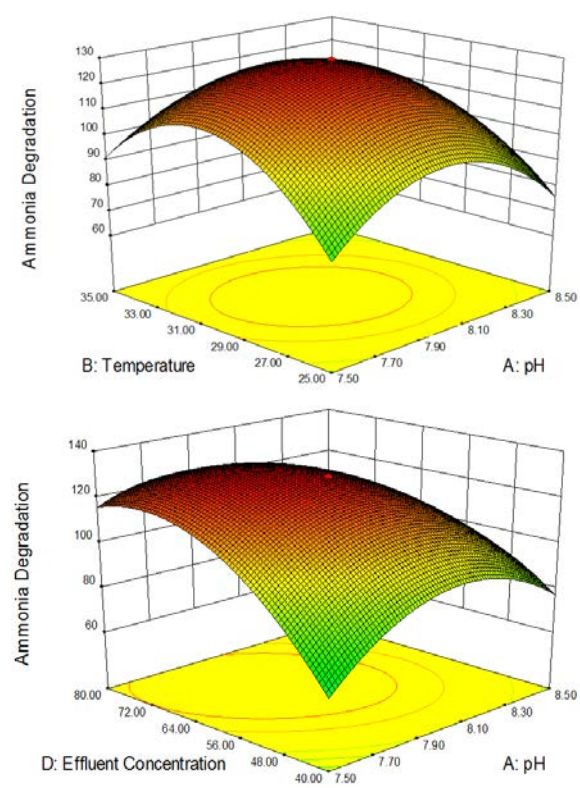


Figure 2 : Parity plot between the experimental and predicted values of Ammonia degradation using mixed culture by Central-Composite design



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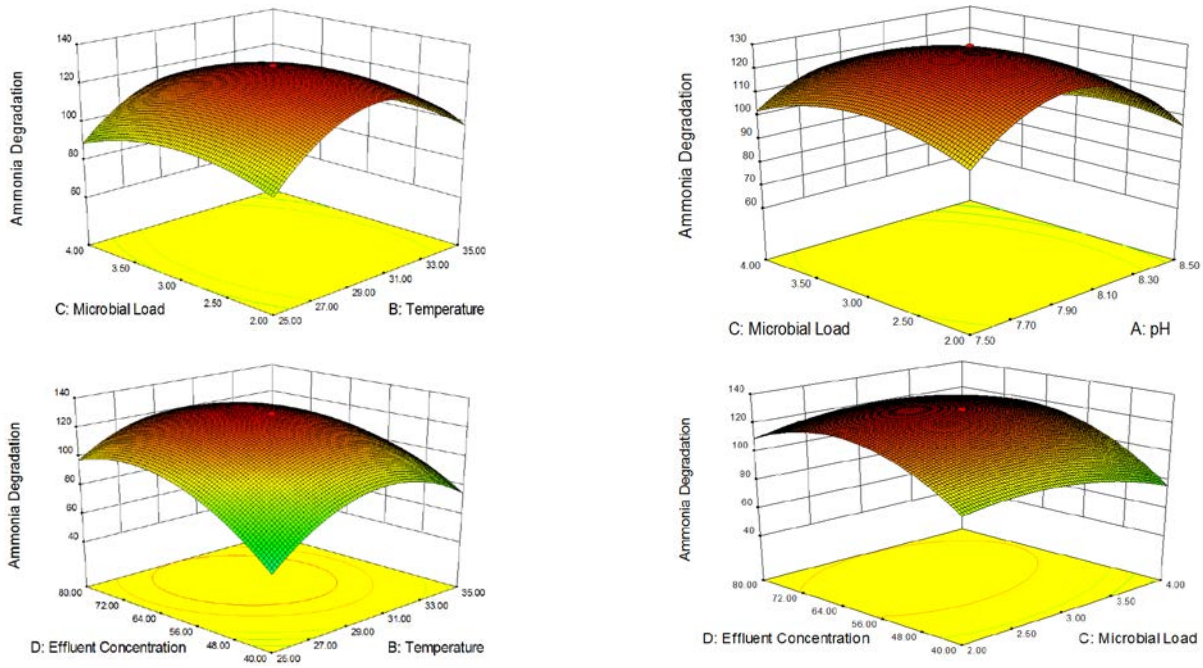

Figure 3.1 to 3.6: Plots showing the interactive effects between significant parameters on ammonia degradation

c) *Batch degradation of waste water in a Rotating Biological Contactor (RBC)*

Degradation of fish processing wastewater was carried out using mixed (*Nitrosomonas* and *Nitrobactor*) culture in a rotating biological contactor under constant operating conditions & optimized parameters. The RBC was operated with an average loading rate of 150mg/L at 15rpm. Ammonia degradation and biomass growth profile are given in Table 5. The result showed that the ammonia concentration decreases continuously with time and reaches a minimum value of 15mg/L on the twenty first day and no further degradation was observed thereafter. An exponential increase in biomass concentration was observed from the sixth day and the stationary phase was attained from the twenty first day. The maximum ammonia degradation (90%) and biomass concentrations (92g/L) were attained and the results were illustrated in Figure 4.

$$\frac{dX}{dt} = kX(X_s - X)$$

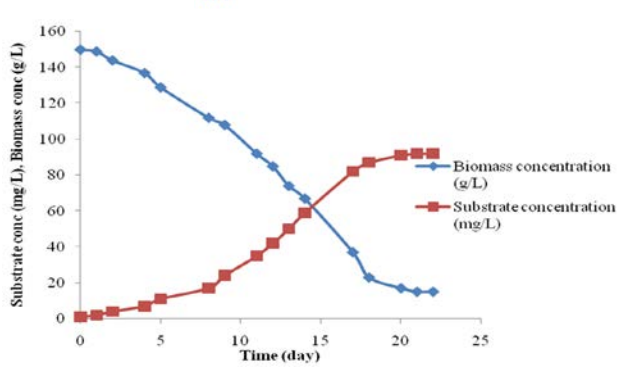

Figure 4 : Time profile of substrate and cell mass concentration in RBC

Table 5 : Time profile of ammonia degradation in RBC

Time(Day)	Ammonia degradation (mg/L)	Biomass concentration (g/L)	% degradation
0	150	1	0
1	149	2	.6
2	144	4	4
4	137	7	8.6
5	129	11	14
8	112	17	25.3
9	108	24	28
11	92	35	38.67
12	85	42	43.33
13	74	50	50.67
14	67	59	55.33
17	37	82	75.33
18	23	87	84.67
20	17	91	88.67
21	15	92	90
22	15	92	90

d) *Growth kinetic models*

i. *Logistic model*

The logistic model states that the rate of growth of the cell is proportional to the cell mass concentration present at any time. When the cell mass reaches the stationary phase there is no growth and hence the rate becomes zero. The growth rate thus depends on how far the cell mass concentration is away from the stationary phase.

Where, k is a constant defined as the specific growth rate. This equation implies that the growth rate increases with an increase in cell mass concentration and is independent of the substrate concentration. Logistic model obtained by rearranging this equation governs the growth of the cell in reality by a hyperbolic relationship and the equation is given by,

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$$\frac{dX}{dt} = k\left[1 - \frac{X}{X_S}\right]X$$

Let $\beta = 1/X_S$, then this equation becomes

$$\frac{dX}{dt} = k[1 - \beta X]X$$

Integrating the above equation from $X = X_0$ at t=0 to X=X at t=t gives a sigmoidal variation of X(t) that may empirically represent both the exponential and stationary phase and is given by,

$$X(t) = \frac{X_0 e^{kt}}{1 - \beta X_0(1 - e^{kt})}$$

This Equation is used to represent cell growth for the batch kinetics, where X_0 is the initial cell mass concentration, g/L and X is the cell mass concentration at any time, g/L.

ii. *Substrate Utilization Kinetics*

Substrate consumption depends on the magnitude of three sink terms, the instantaneous cell mass growth rate, the instantaneous product formation rate and a cell mass maintenance function. The kinetic model is given by

$$S = S_0 - \left[\frac{X - X_0}{Y_{X/S}}\right]$$

The time profile of biomass growth for mixed nitrifying cultures is represented in Table 6. The cell growth kinetic model given in Table 7 is used to represent the batch kinetics. The experimental data for biomass growth was fitted in to the above equations for mixed cultures. Using MATLAB the k and β values of the mixed culture were found to be 0.5284 day^{-1} and 0.0114 L/g with a correlation coefficient of 0.9974 as shown in Table 8.

Table 6 : Time profile of substrate utilization and cell mass growth

Time(Day)	Cell mass concentration (g/L)	Substrate concentration (mg/L)
0	1	150
1	2	148
2	4	146
3	7	142
4	11	138
5	13	133
6	20	125
7	28	113
8	35	99
9	48	82
10	60	73
11	73	58
12	79	49
13	82	41
14	84	36
15	86	30
16	87	25
17	88	23
18	88	21
19	88	21

Table 7 : Batch kinetic models used for cell growth and substrate utilization

Model	Equations
Logistic	$X(t) = \frac{X_0 e^{kt}}{1 - \beta X_0(1 - e^{kt})}$
Substrate utilization	$S = S_0 - \left[\frac{X - X_0}{Y_{X/S}}\right]$

Table 8 : Values of kinetic parameter and regression coefficient (R^2)

Model	Parameter estimation	Regression coefficient (R^2)
Logistic	k=0.5284 day^{-1} β= 0.0114 L/g	0.9974
Substrate utilization	$Y_{X/S}$=0.7162	

e) Model evaluation

Various growth and substrate utilization kinetic models were analyzed using the experimental data for mixed nitrifying cultures. The models which showed maximum fit are represented the previous table 8 along with the estimated parameters. The other models and the estimated parameters obtained for those models during the biodegradation of ammonia by mixed nitrifying cultures were given in Table 9.

The optimized model parameter values are evaluated using MATLAB coding. With the estimated parameter values obtained using the respective models, the profiles of cell mass growth and substrate utilization are simulated for the entire time period of fermentation. Comparisons are made between the experimental data and the theoretical predictions as shown in Figure 5.1 to 5.6 for mixed nitrifying culture. The regression coefficient values (R^2) were estimated for the substrate concentration and are presented in Table 9 mixed cultures. From the regression coefficient values, it is clear that the correlation between the experimental data and the theoretical predictions are good. Thus, the Logistic model represents ammonia degradation very well for the mixed culture of *nitrobactor* and *nitrosomonas.*

Table 9 : Growth kinetic parameter and regression for different models

Model	Parameter estimation	Regression coefficient (R^2)
Monod model	μ_m = 0.5875 k_s = 124.1	0.4290
Herbert model	μ_m =0.5875 k_s =52.1 m = 0.2768	0.5195
Shehata & Marr model	μ_m =0.5875 μ_1 = - 80.54 k_1 =10.29 k_2 = 10.57	0.4618

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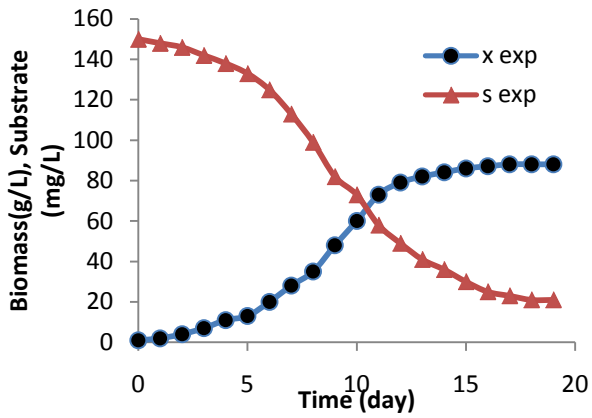
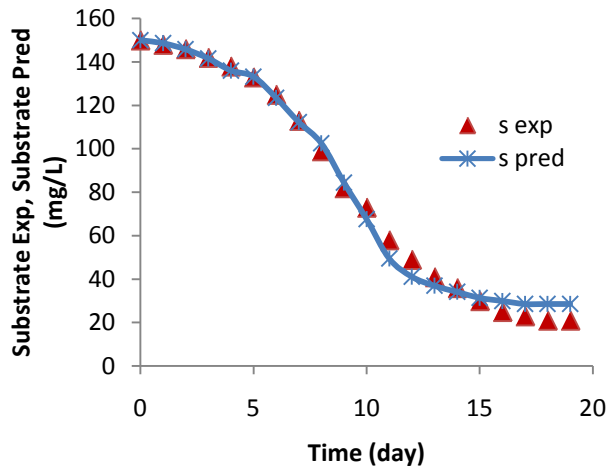
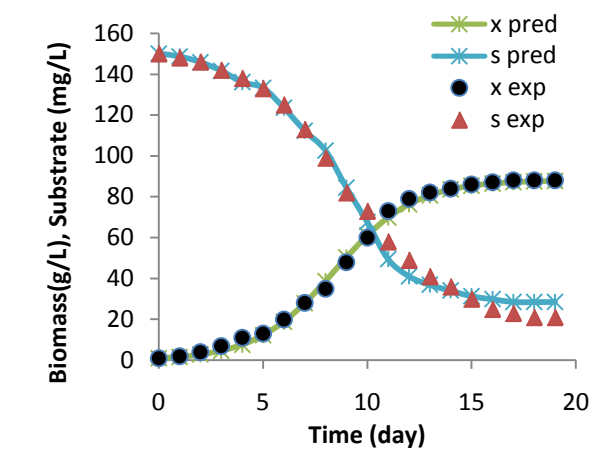
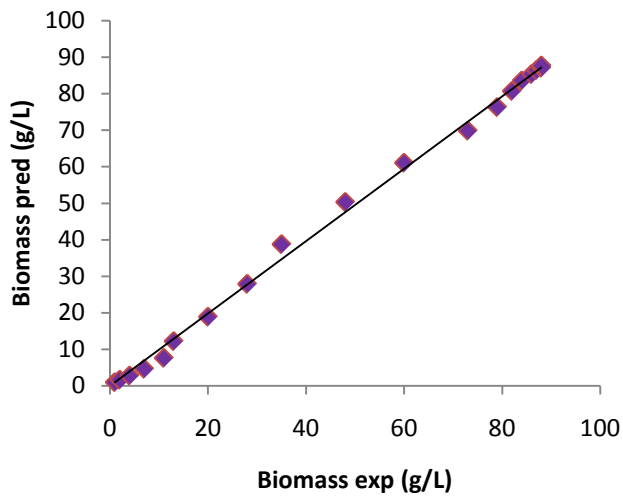
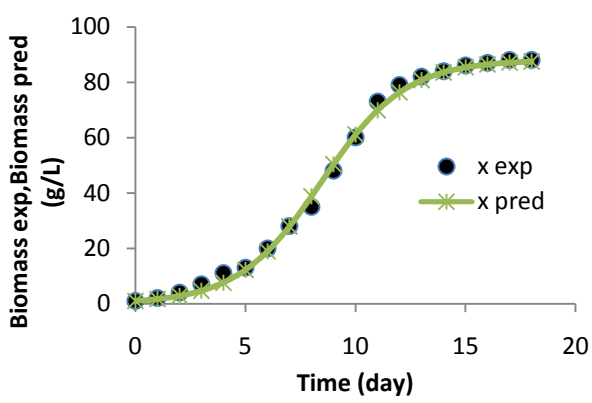
Tessier model	μ_m =0.5875 T = 0.8098	- 3.8740
Haldane model	μ_m = 0.5875 k_i = - 0.0007875 k_s = 132.8	0.4309











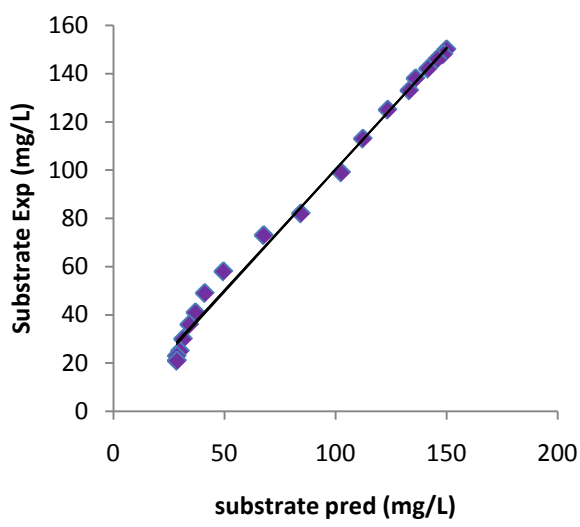


Figure 5.1 to 5.6 : Comparison between experimental data (points) and theoretical predictions (lines) of ammonia degradation

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IV. Conclusion

The present work demonstrated the nitrification of fish processing waste water using a mixed culture of *Nitrosomonas* and *Nitrobactor*. The process parameters namely, the pH, temperature, microbial load and effluent concentration were optimized using Central Composite Design of RSM. The optimum conditions were found to be pH:8; temperature: 30°C; microbial load:3%(v/v) and effluent concentration:60% for mixed culture. The physical-chemical characteristics of fish processing wastewater were analyzed and it showed high values of BOD (9003 mg/L) and COD (11040 mg/L). The batch kinetic data obtained for mixed cultures were fitted into various models, namely, *Logistic, Monod, Herbert, Shehata & Marr, Tessier and Haldane* models. Among these models the Logistic model fits the data best with a correlation coefficient of 0.9974 and 0.9994 for mixed cultures of *nitrobactor* and *nitrosomonas*. Maximum ammonia degradation was observed for mixed culture, where Rotating Biological Contactor was operated on a batch basis with an average organic loading rate of 150mg/L of ammonia at 20 rpm and the experimental data were collected. The result showed that, a maximum ammonia degradation of 135mg/L was observed on twenty second day. The future research has been suggested for treating the fish processing waste water using the isolated nitrifying bacteria from the soil.

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Table 3 : Five level Central-Composite design matrix for the optimization of significant process parameters in ammonia degradation using mixed culture of *Nitrobactor* and *Nitrosomonas*

Run No.	pH	Temperature (°C)	Microbial Load % (v/v)	Effluent Concentration (%)	Ammonia degradation mg/L	
					Experimental	Predicted
1	1	-1	1	1	52	49.167
2	1	-1	1	-1	27	26.750
3	-1	1	1	-1	35	35.083
4	0	0	0	0	129	129.000
5	2	0	0	0	23	23.083
6	1	1	1	1	20	21.583
7	-1	-1	-1	-1	15	14.917
8	0	-2	0	0	17	18.083
9	-1	-1	1	-1	9	9.667
10	1	1	-1	-1	55	54.917
11	0	2	0	0	22	21.417
12	0	0	0	0	129	129.000
13	0	0	0	2	60	62.417
14	0	0	-2	0	90	90.750
15	0	0	0	0	129	129.000
16	-1	-1	1	1	84	85.583
17	1	1	1	-1	28	29.667
18	1	-1	-1	-1	46	46.500
19	-1	-1	-1	1	75	71.333
20	1	1	-1	1	30	27.333
21	0	0	2	0	80	79.750
22	-1	1	1	1	83	80.500
23	-1	1	-1	-1	45	45.833
24	1	-1	-1	1	48	49.417
25	-2	0	0	0	50	50.417
26	0	0	0	-2	16	14.083
27	0	0	0	0	129	129.000
28	-1	1	-1	1	70	71.750
29	0	0	0	0	129	129.000
30	0	0	0	0	129	129.000
31	0	0	0	0	129	129.000

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Table 4 : Results of the ANOVA of the process parameter optimization data for ammonia degradation using mixed culture by Central-Composite design of experiments

Source	Coefficient	Sum of Squares	Degrees of Freedom (DF)	Mean Square	F Value	P-Value Prob > F
Model	129.00	48353.80	14	3453.84	849.31	0.0001
A-pH	-6.83	1120.67	1	1120.67	275.57	0.0001
B-Temperature	0.83	16.67	1	16.67	4.10	0.0611
C-Microbial load	-2.75	181.50	1	181.50	44.63	0.0001
D-Effluent concentration	12.08	3504.17	1	3504.17	861.68	0.0001
AB	-5.63	506.25	1	506.25	124.49	0.0001
AC	-3.63	210.25	1	210.25	51.70	0.0001
AD	-13.38	2862.25	1	2862.25	703.83	0.0001
BC	-1.38	30.25	1	30.25	7.44	0.0156
BD	-7.63	930.25	1	930.25	228.75	0.0001
CD	4.87	380.25	1	380.25	93.50	0.0001
A^2	-23.06	14588.68	1	14588.68	3587.38	0.0001
B^2	-27.31	20460.96	1	20460.96	5031.38	0.0001
C^2	-10.94	3281.25	1	3281.25	806.86	0.0001
D^2	-22.69	14118.11	1	14118.11	3471.67	0.0001
Residual		61.00	15	4.07		
Lack of fit		61.00	10	6.10		
Pure error		0.000	5	0.000		
Total		48414.80	29			

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Engineered Bovine Serum Albumin Nanoparticles Incorporated Alpha Amylase Preparation for the Improved Activity

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Keywords: nano albumin, amylase, nanosphere, enzyme activity.

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S. Karthick Raja Namasivayam [α], Sasi kumar [σ] & Shaleesha Stanley [ρ]

Abstract- In the present study, the improved enzyme activity of alpha amylase encapsulated with bovine serum albumin nanoparticle (BSA) was carried out. Initially. The method for preparation of BSA nanoparticles was optimized with various parameters such as pH, acetone to BSA ratio, cross linking time and concentration of BSA. The optimal pH was found to be 4.5, 10 mg/ml of BSA concentration, acetone to albumin ratio was to be 4.5, cross linking time of 3hrs which gives the higher yield of BSA nanoparticles. The Nano-enzyme conjugate was prepared using the optimized conditions. The encapsulation of α-Amylase Enzyme with BSA Nanoparticle was done based on hydrophobic ion pairing (HIP) complexation. And the Scanning electron micrographs of BSA nanoparticle encapsulated enzyme at the targeted size of 100nm. Distinct changes in the kinetic parameters of nano albumin encapsulated α-amylase was observed. Enzyme activity of nano encapsulated alpha amylase was found to be maximum at the pH of 8.0,temperature 80ºC and 4.5mg/ml of substrate concentration.

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I. Introduction

Nanotechnology has been introduced into several aspects of the food science, including encapsulations and delivery systems, which protect and deliver functional ingredients. Bioactive ingredients such as nutrients, phytochemicals, nutraceuticals, drugs and enzymes may be incorporated into nanoparticles to maximize delivery efficiency and increase desirable benefits (Arnedo et al, 2002). Since nanoparticles are submicron and sub-cellular in size, they have versatile advantages such as increased surface area and reactivity, increased gastric residence time and permeability, and improved solubility in both aqueous and organic phases. Brownian motion can provide enough energy to keep exceptionally small particles agitated and hence precipitation is less likely to happen with nanoparticle suspension. Therefore, suspensions of nanoparticles are easier to stabilise because precipitation is less likely Bovine serum albumin (BSA) was chosen as the material for the particle matrix. BSA has great potential as a nanocarrier in food and pharmaceutical applications. BSA is non-toxic and degradable in vivo, so the nanoparticles generated by using it are easily adaptable to the human body (Jahanshahi et al,2008, Langer et al,2003, Desai et al,1996).The addition of bovine serum albumin (BSA) enhances stabilization both by protection against protease and by stabilization in the low concentration-regime protein.

Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view. In particular, a greater awareness of conservation issues has forced industries to consider alternative, cleaner method. With this regard, the use of enzymes as industrial catalyst is becoming the best option, and enzymes are gradually replacing chemical catalysts in many areas of industry (Karthick Raja Namasivayam and Nirmala,2011).The major classes of enzyme offering immediate application are the hydrolytic enzyme (which account approximately 75% of the industrial enzymes produced (Prasanna ,2005).Through the use of hydrolytic enzymes many different natural and agricultural polymers can be processed and up graded for eventual human or animal consumption, or for further bioconversion in to value added products The ability to commercially produce sugars from starch using amylolytic enzymes is an example of such processes (Ivo safari,1989).

Due to the increasing demand for enzymes in various industries, there is enormous interest in searching for enzymes suitable for application, and their cost effective production techniques (Pandey et al, 1999). α -Amylases are starch degrading enzymes. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolyzing a-1, 4-glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Sodhi et al, 2005, Vishwanathan and Surlikar, 2001). In the present study, nano formulation of alpha amylase with BSA nanoparticles and the improved enzyme activity of nano encapsulated enzyme was studied.

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II. Materials and Methods

a) Preparation of BSA nanoparticles

Simple coacervation technique was implemented for preparation of BSA nanoparticles (Muller et al, 1996). Anhydrous ethyl alcohol was added to 150 ml BSA (5 mg/l in 10 mM Tris/HCl contained 0.02% sodium azide) till the solution became turbid then 150 μl of 25% glutaraldehyde was added for cross linking. The reaction was continued at room temperature (24°C). Ethanolamine was added to block the non-reacted aldehyde functional group. Also Tween-20 was added at a final concentration of 0.01% (v/v) to stabilize the preparation. The suspension is then ultrasonicated for 30 mins. Large aggregates were eliminated by centrifuge (50,000 g, 30 min, 4°C). The pellet is then lyophilized to form fine powder.

i. *Characterization of nanoparticles*

The morphologies of the BSA nanoparticles were observed by scanning electron microscopy (SEM) The sample was sputtered with palladium gold for 30s under Polaron machine (BAL-TEC, Model SCDOOS, Switzerland). Afterwards, SEM was performed with a Carl Zeiss supra 55 (Germany) Field emission scanning electron microscope with the upper detector at 15 kV. The magnification was set at 65,000.

b) Optimization of the nanoparticles preparation

i. *Determination of optimal pH*

The optimization of BSA nanoparticles synthesis was performed over a pH range between 6 and 10. For the analysis, the pH value of the suspension was automatically adjusted by the titration unit by addition of 0.1N hydrochloric acid or 0.1N sodium hydroxide solution, respectively. At 5 predefined pH values between 6 and 10, the yield percentage of the nanoparticles was measured and the particle size was determined by SEM.

c) Determination of optimal acetone to Albumin ratio

The optimization of the BSA nanoparticles preparation based on the acetone concentration used for coacervation process. Ethanol concentration used for the desolvation influences the yield and the particle size of the nanoparticles. Five different ethanol to album in ratios were preselected using earlier works on BSA nanoparticles synthesis. The different ratios include 2:1, 2. 5:1, 3:1, 4:1 and 5:1.optimal ethanol concentration was determined based on the particle size studied using SEM and yield percentage.

d) Determination of Crosslinking Time

The crosslinking of the acetone coacervates takes place after the addition of glutaraldehye. The crosslinking time influences the particle size and yield percentage of the nanoparticles. The cross linking time at a range from 6h to 16h was predetermined and the optimal crosslinking time for the maximum synthesis was determined.

e) Encapsulation of α-Amylase with BSA Nanoparticle

Alpha amylase (analytical grade) was obtained from SRL laboratories as powder form with 60 mu/g enzyme activity and the nano encapsulation with BSA nanoparticles was carried out by simple coacervation method that followed in free BSA nanoparticles synthesis. Analytical grade ethanol was added at a rate of 2ml for every 5 mins to 20 ml of 1% BSA by intermittent method (prepared in deionized water & the pH 9 was adjusted with 0.2M NaoH) and stirrer continuously in magnetic stirrer at 500 rpm till the solution became just turbid. Then 100 μl of 25% glutaraldehyde was added for cross linking. The reaction was continued at room temperature (24°C) for 3 hours. Ethanolamine (100μl) was added to block the non-reacted aldehyde functional group. 10mg of α-amylase was added to the medium and stirrer continuously for 1 hour. Also Tween-20 was added at a final concentration of 0.01% (v/v) to stabilize the preparation. The suspension was then ultracentrifuged (20,000g, 30minutes) for two cycles. The pellet was then lyophilized or keep it in hot air oven for 20 minutes at 55˚C to form fine powder of encapsulated α-amylase enzyme(Enz-A).Morphology of the encapsulated enzyme was characterized by scanning electron microscopy (SEM).

f) Enzyme assay

The activity of Enz-A was assayed using a reaction mixture comprising of 1ml encapsulated enzyme,1ml of 1% (w/v) soluble starch solution in 0.05 m citrate buffer solution (pH 4.5).The reducing sugars liberated were estimated by the 3, 5 Dinitrosalicylic acid (DNS) method. The reaction mixture was incubated at 60˚C for 20 minutes and the reaction was terminated by adding 2ml of DNS in the reaction tube and then immersing the tube in boiling water bath (60˚C) for 15 minutes. The absorbance was measured at 540 nm with spectrophotometer. The Enz-A activity was defined as by the following formula

$$\text{Amylase activity (IU/ml/min)} = \frac{\text{Amount of sugar released x 1000}}{\text{Molecular weight of maltose x Time of incubation}}$$

The enzymatic assay result was compared with the free and the immobilised enzyme assay data obtained from the literature to get the efficiency rate of the encapsulated enzyme (Enz-A).

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g) Evaluation of improved enzyme activity of encapsulated enzyme.

i. *Effect of pH*

1ml of the free, immobilized and encapsulated enzyme was incubated with buffer with pH of 3, 4, 5, 6,7,8,9 and 10 respectively at 32°C for 10 minutes and the enzyme activity was determined.

ii. *Effect of Temperature*

1ml of the free, immobilized and encapsulated α-amylase enzyme was incubated with different temperature ranged from 30°C, 40 °C, 50 °C, 60 °C, 70°C, 80°C, 90°C and 100°C respectively for 10 minutes. The enzyme assay was carried out as described earlier.

iii. *Effect of substrate concentration*

1ml of the free, immobilized and encapsulated enzyme was incubated with different concentration of starch solution (1 to 10mg/ml), incubated at 32°C for 10 minutes and the enzyme activity was determined.

h) Kinetic parameters of encapsulated enzyme

0.5 ml aliquot of starch solution(100- 1000 mg/ml) was mixed with equal volume of free, imkobilized and encapsulated enzyme ,incubated 32°C for 10 minutes and 1ml of GOD-POD reagent was added.

III. Result and Discussion

The strategy to control size was to adjust BSA concentration, pH, ionic strength, crosslinking reaction rate and agitation speed in the desolvating conditions. BSA nanoparticles formed in different sizes at the above conditions since the desolvation process was influenced by the electrostatic attraction and repulsion among BSA molecules (Fig 1 to 8 a).

a) Determination of optimal pH

Optimisation of pH condition for BSA nanoparticle at the target size of 100nm plans are shown in table 1.Size control of BSA nanoparticles was conducted by modifying desolvation methods. The particle size was controlled by adjusting pH which affects the coagulation of the BSA molecules. The isoelectric point (pI) of BSA is about 4.9. When pH shifts toward the pI, the enhanced protein–protein interactions increase coagulation among BSA molecules, larger BSA particles was formed. On the other hand, the enhanced protein–solvent (or water) interactions decrease coagulation when pH was far from the pI. pH 9 provides a highly electrostatic repulsive condition for the BSA molecules and concomitantly, coagulations by protein–protein interactions are limited, as a result in table 1,2, fine BSA particles with required target mean size was formed.

b) Determination of optimal Acetone to Albumin ratio

Optimization of volume consumptions of acetone for the BSA nanoparticle preparation at target size of 100nm.The amount of acetone for the 100 nm targeted BSA nanoparticle preparation was 35 ml. Consumption of acetone was inversely proportional to the targeted size.

c) Determination of Crosslinking Time

Crosslinking incubation time on BSA nanoparticle preparation was optimised as in table 1. It was Influences the cross linking processes; as a result different mean size of BSA nanoparticles was formed. The target size of 100nm was achieved for 3hrs incubation from source 4)

d) Determination of optimal BSA concentration

The effect of BSA concentration on BSA nanoparticle size was conducted as the concentration of BSA in creased the particle size was also increased. The target particle mean size of (115.6nm) was achieved at the concentration of 10mg/ml.

e) Determination of optimal agitation rate

Agitation speed that in terfere with the target size of the BSA nanoparticle, mean size of particle (115.6 nm) close to the target size was obtained at agitation speed of 500 rpm.

f) Surface-area-to-volume-ratio of BSA nanoparticles

The greater surface-area-to-volume-ratio of BSA nanoparticles particle than rest of the sources was found in source 4 parameter. Surface area is a material property of particles that is often a determining factor in bioavailability, dissolution rate, adsorption, catalyst activity, and toxicity (Oberdorster et al, 1996).The surface area is a more useful and accurate metric than concentration or dosage, which is traditionally believed to be the most important .The concept of total surface area can be used to describe the surface area in a sample. One such is the surface-area-to-volume-ratio, and is more useful than the concentration or the mean diameter of particles. The surface-area-to-volume ratio is the amount of surface area per unit volume of particles. The surface-area-to-volume ratio can be calculated using size distribution data of particles under that all particles are spheres. Table 2 shows the surface-area-to volume-ratios of BSA nanoparticles of target size 100 nm. The particles for the 100 nm plan obtained the largest surface area in source 4, whereas those from sources 1, 2, 3, & 5 had a relatively small surface area. In any case, the surface-area-to-volume-ratio can be used to represent a major physical property of the particles instead of diameter-related values Furthermore; the surface-area-to volume-ratio is useful for comparative studies of nano-scaled materials.

g) Encapsulation of α-Amylase Enzyme with BSA Nanoparticle.

The encapsulation of α-Amylase Enzyme with BSA Nanoparticle was done based on hydrophobic ion pairing (HIP) complexation. And the Scanning electron micrographs of BSA nanoparticle encapsulated enzyme

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(Enz-A) at the targeted size of 100nm by Intermittent Method was shown in figure 9.

h) Kinetic Analysis for Encapsulated (Enz-A)

Kinetic parameters of Enz-A with the free and immobilized α-amylase were measured. For these forms of α-amylase enzyme activity, Michaelis-Menten type kinetic behavior was observed. The Km and V max values as determined from the figure 10 found to be 3.2 mg/ml and $1.89x10^{-3}$ μ mole/ml. s (for Enz-A), 3.8 mg/ml and $1.46x10^{-3}$ μ mole/ml. s (for free enzyme, 5.9mg/ml and $1.58x10^{-3}$ μ mole/ml. s. Therefore, for Enz-A form of α-amylase Km value was decreased and the V max was increased as compared to free and immobilised form. The Km value shows the affinity of enzyme for its substrate. Lower the Km value more is the affinity of enzyme for its substrate.

i) Effect of pH

Effect of pH on activity of Enz-A, free and immobilized α-amylase was given in fig 11.Optimum pH values were 8, 7 and 6 for Enz-A, Free and immobilized α-amylase respectively. This shift in optimum pH could be resulted from the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site. The pH is one of the major parameters capable of shifting enzyme activities in reaction mixture, encapsulation and Immobilization usually results in shift of optimum pH due to conformational changes in enzymes

j) Effect of Temperature

The activity of enzyme is also strongly dependent on temperature. The activity of Enz-A, free, Immobilised α-amylase increased with temperature and maximum activity was observed at 80°C,50°C,60°C as shown in fig.12.The optimum temperature of α-amylase was shifted to 60°C after immobilization in calcium alginate beads and 80°C for Enz-A. As was evident from the fig 12, α-amylase after encapsulation possessed temperature /resistance than the free enzyme and Immobilised enzyme

k) Effect of substrate

The activity of Enz-A, free, Immobilised α-amylase enzyme based on substrate concentration was varied with respect to Km value as shown in fig.13.. The increased activity facilitated by Enz-A within low substrate concentration than the Free and immobilised α-amylase enzymes was observed. The Km value was furnished with low numbers represents strong activity even in low concentration of substrate was observed The optimum activity with respect to substrate concentration for Enz-A, free and Immobilised α-amylase was 4,5 and 8 mg/ml

l) Effect of Incubation Time

The enzymatic activity of Enz-A, free, Immobilised α-amylase enzyme was varied with respect to time of incubation. The retained activity facilitated by Enz-A with an incubation time of 14 hrs. followed by immobilised and free α-amylase enzymes at a rate of 12 and 10 respectively The present study clearly revealed the biocompatible bovine serum albumin nanoparticles encapsulated alpha amylase would suggest the possible utilization of enzyme preparation with improved activity.

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Table 1 : Optimization of the parameters for the BSA nanoparticles at the target size of 100nm

S.no	*Parameters*	*Source 1*	*Source 2*	*Source 3*	*Source 4*	*Source 5*
1.	Ph	6	7	8	9	10
2.	BSA in (%)	5	4	3	2	1
3.	Acetone volume(in ml)	21	20	25	35	30
4.	Crosslinking incubation time(in hrs)	15	12	9	3	6
5.	RPM	200	300	400	500	600
6.	Mean size of the particle (in nm)	379.8	352.9	286.4	115.6	130.1

Table 2 : Mean size and surface-area-to-volume-ratio of BSA nanoparticles at the targeted size: 100nm

Target size (nm)	Method	Mean size (nm)	Surface-area-to-volume ratio
100	Intermittent Method	115.06	6.62×10^{10}
	Continuous Method	137.06	1.58×10^{11}

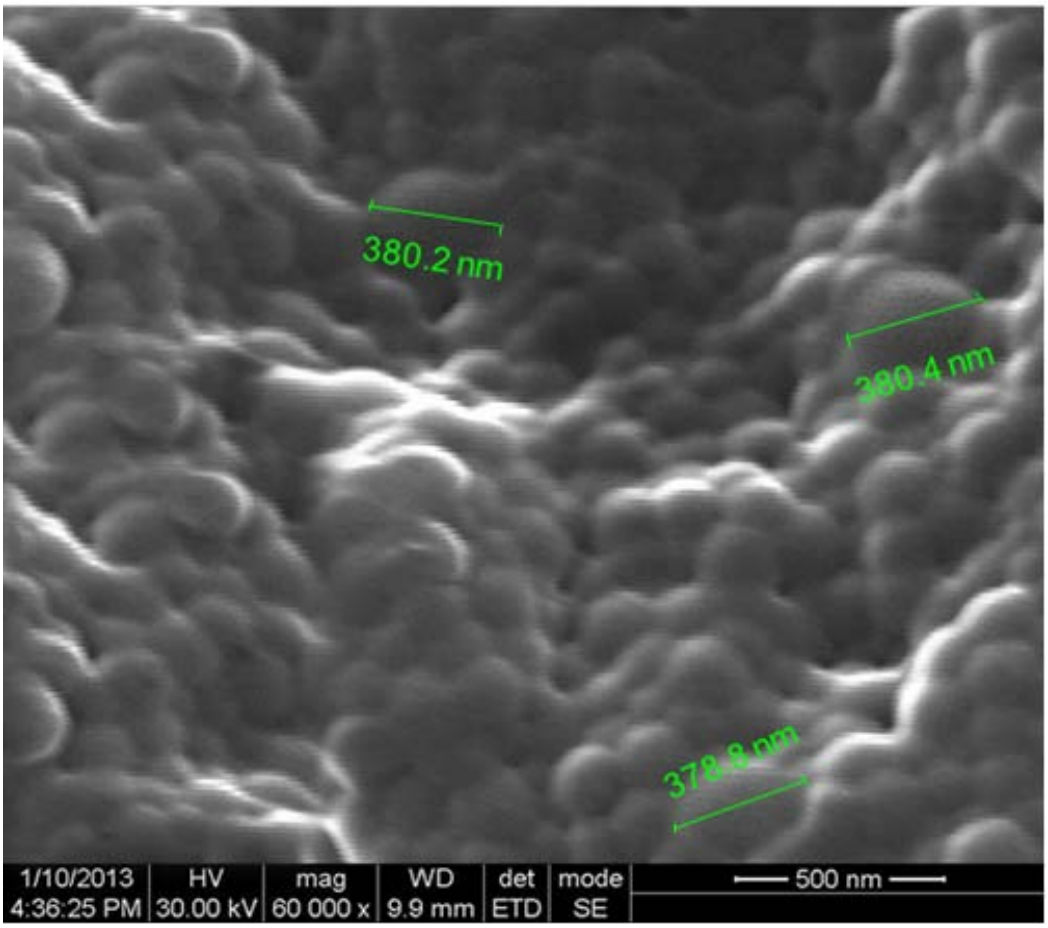

Figure 1 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 1 parameter

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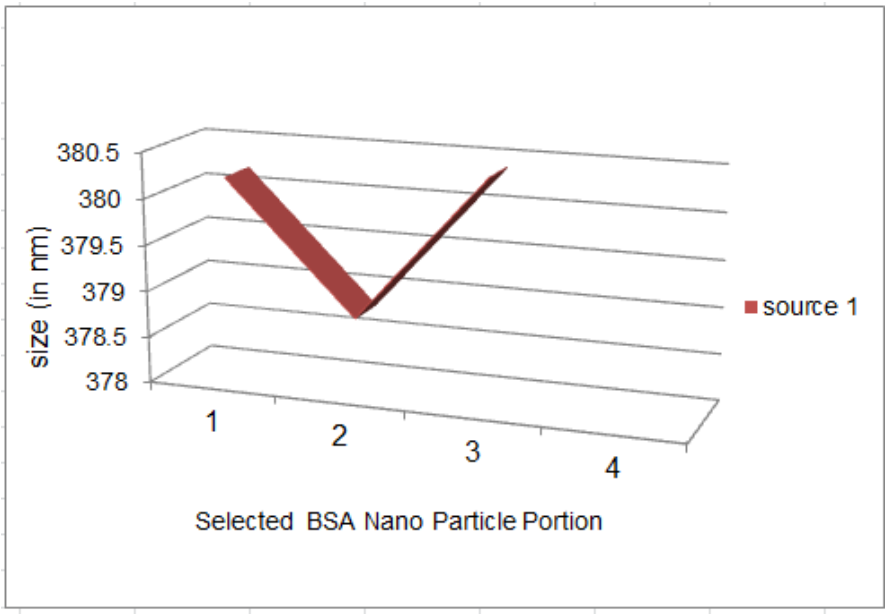

Graph 1 a : The optimisation of size of BSA nanoparticles by source 1 parameter

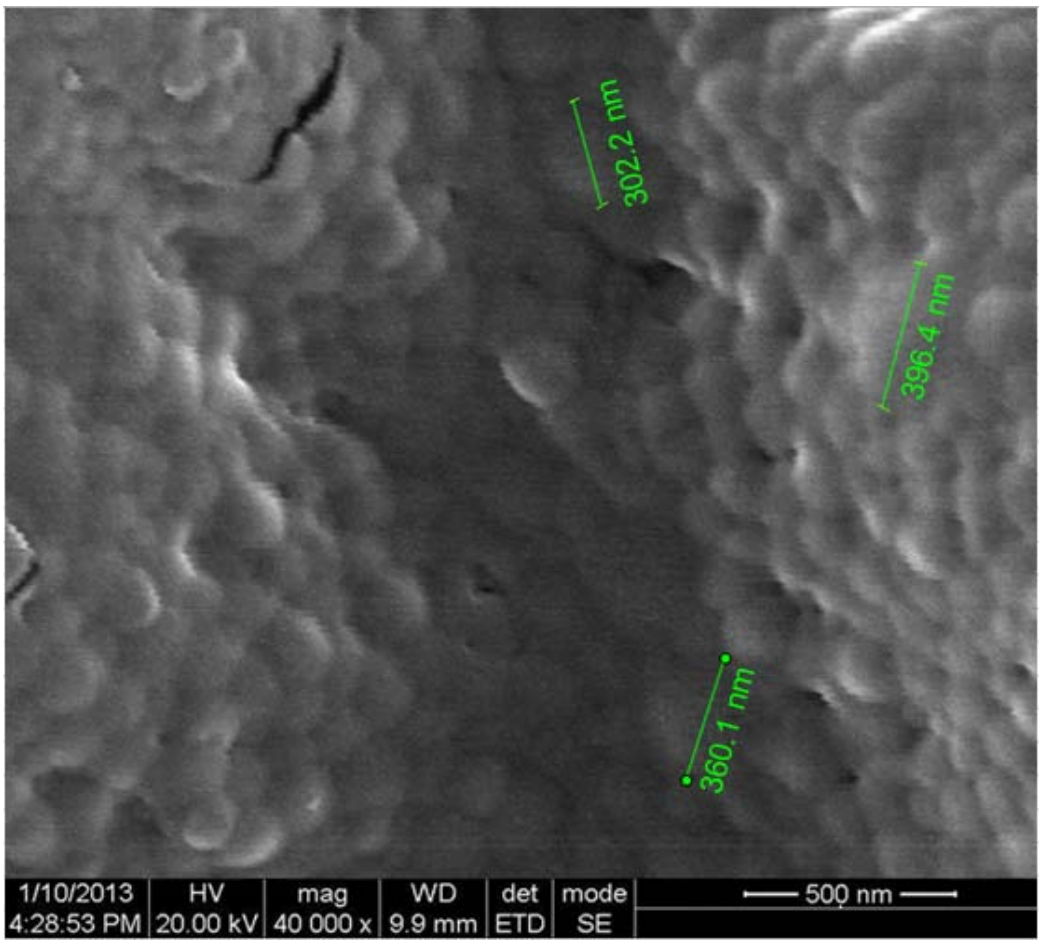

Figure 2 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 2 parameter

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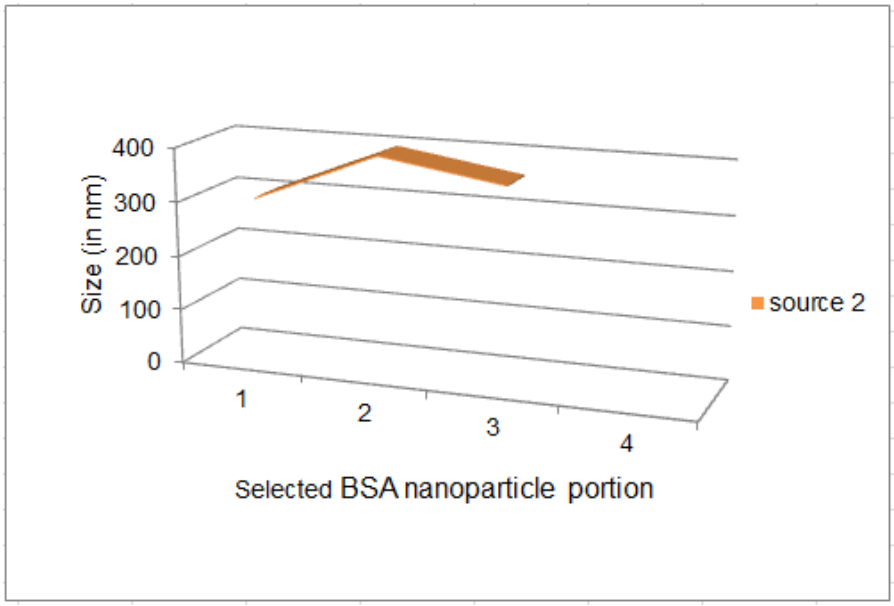

Graph 2 a : The optimisation of size of BSA nanoparticles by source 2 parameter

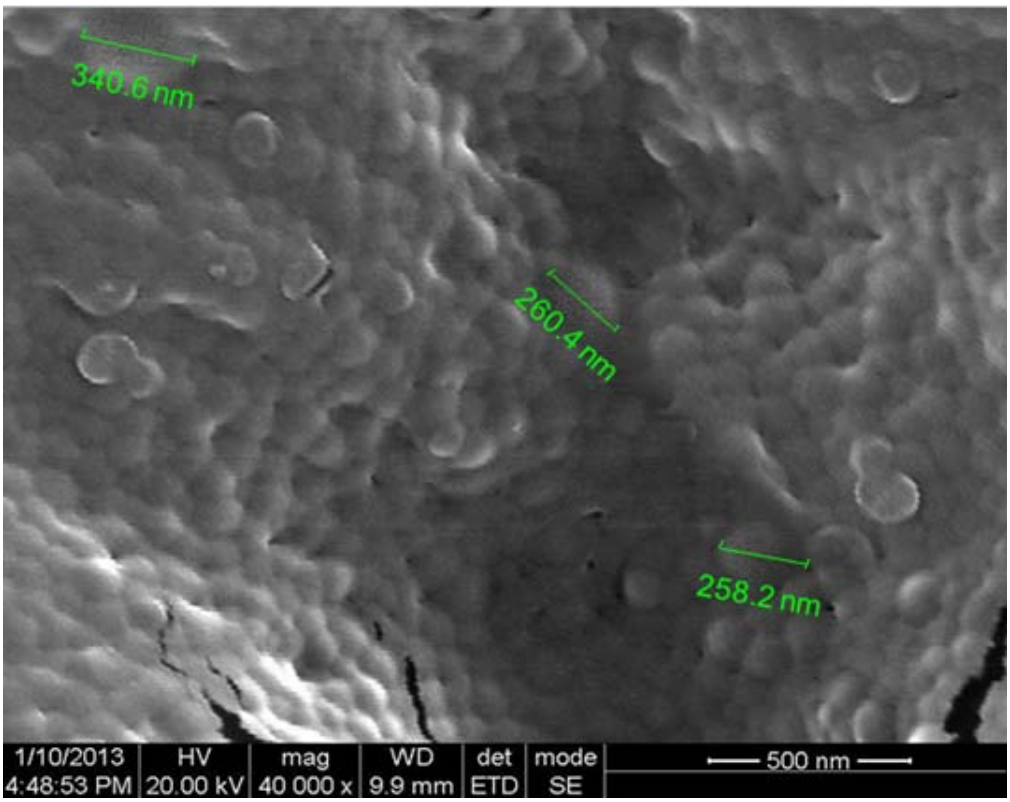

Figure 3 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 3 parameter

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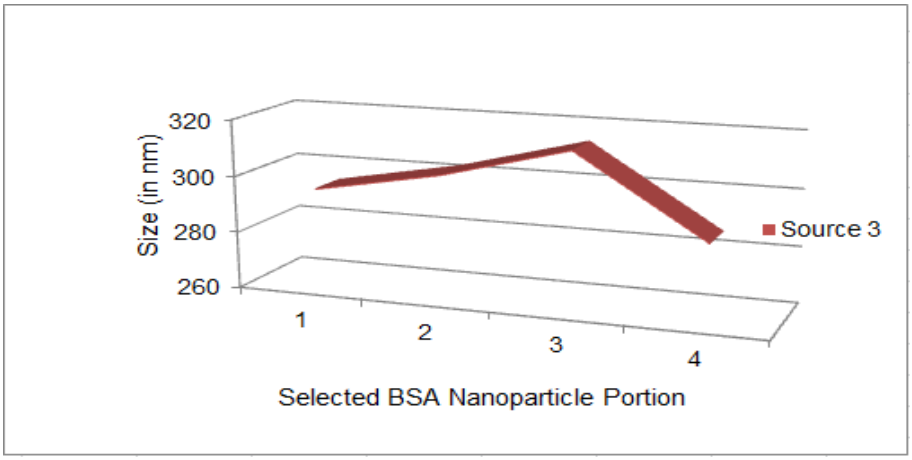

Figure 3 a : The optimisation of size of BSA nanoparticles by source 3 parameter

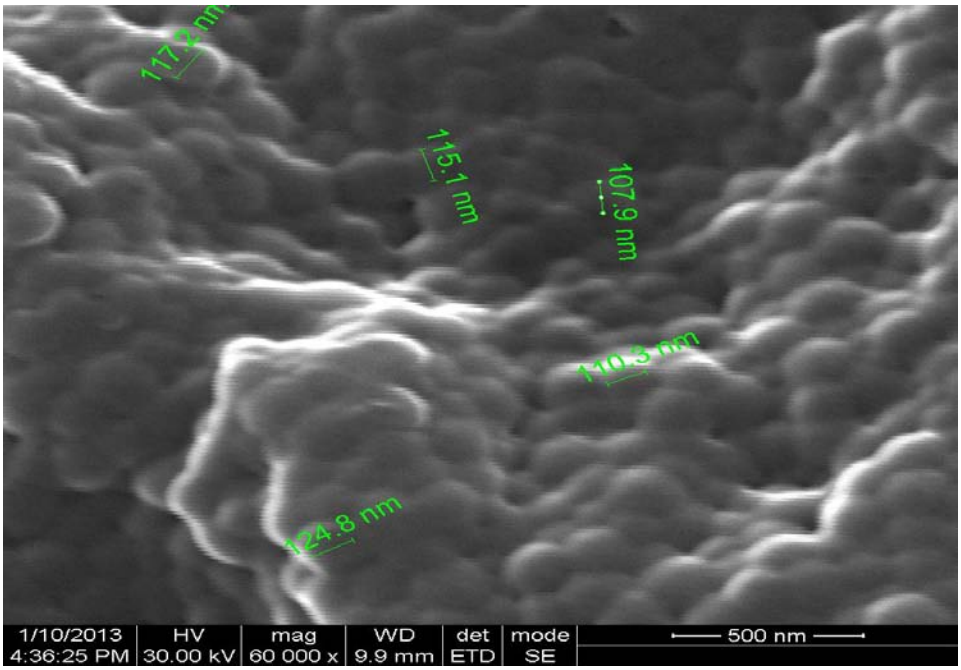

Figure 4 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 4 parameter

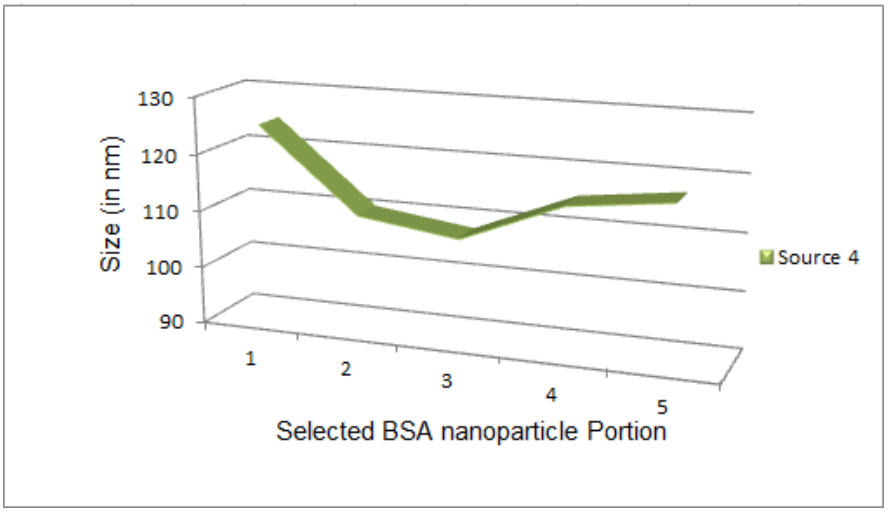

Figure 4 a : The optimisation of size of BSA nanoparticles by source 4 parameter

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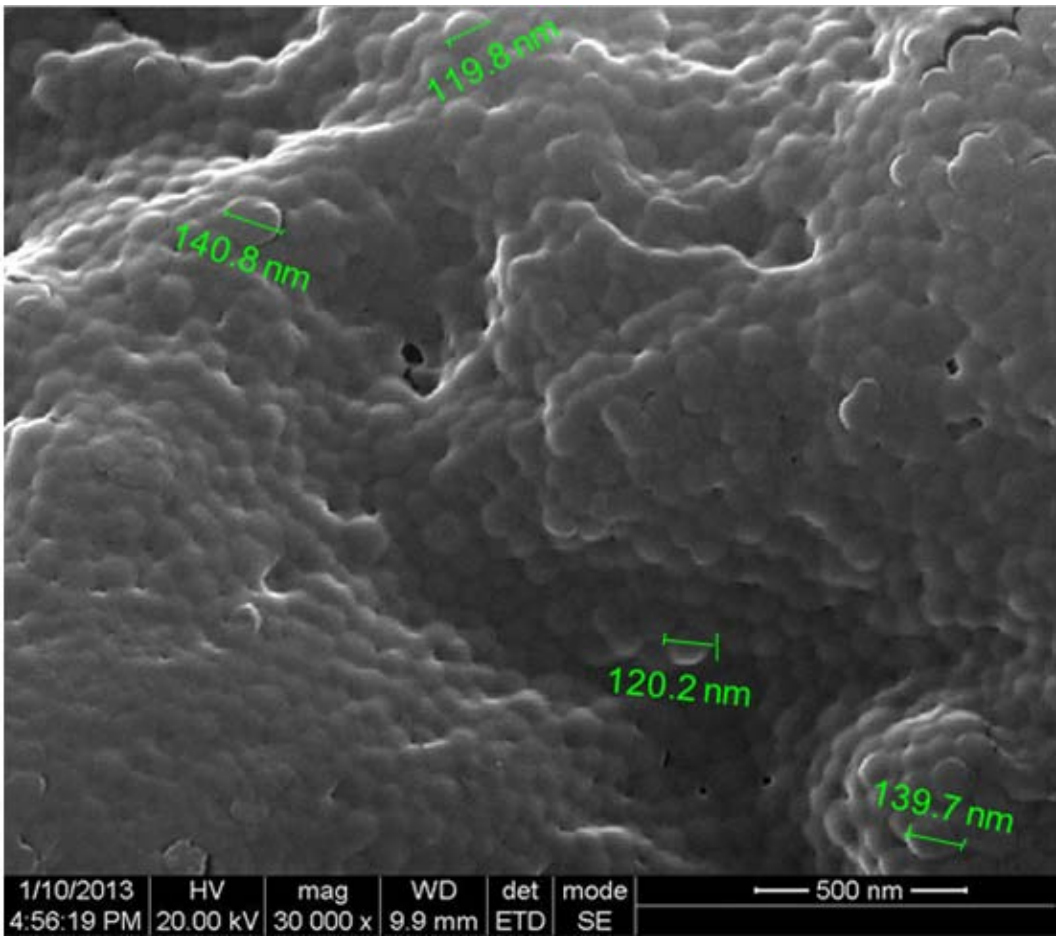

Figure 5 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Source 5 parameter

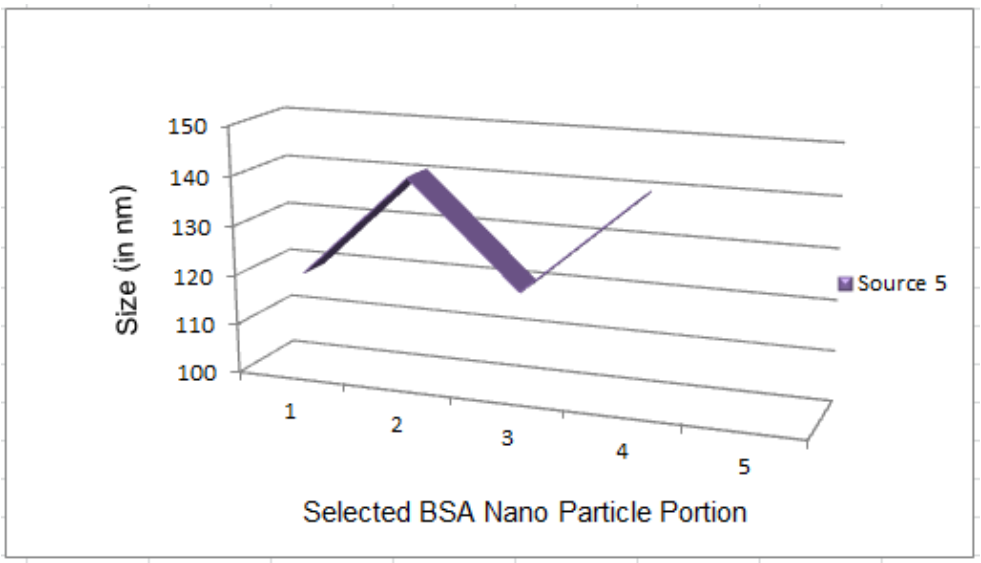

Figure 5 a : The optimisation of size of BSA nanoparticles by source 5 parameter

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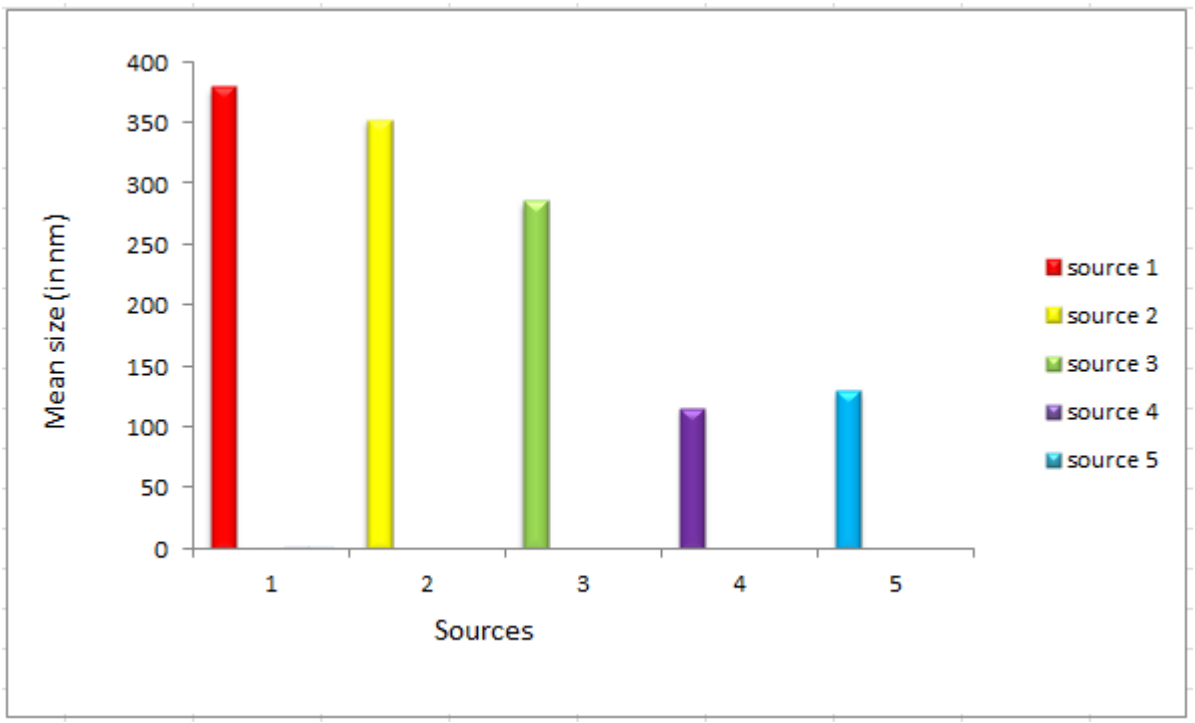

Figure 6 : Mean size of BSA nanoparticles by different sources of parameter

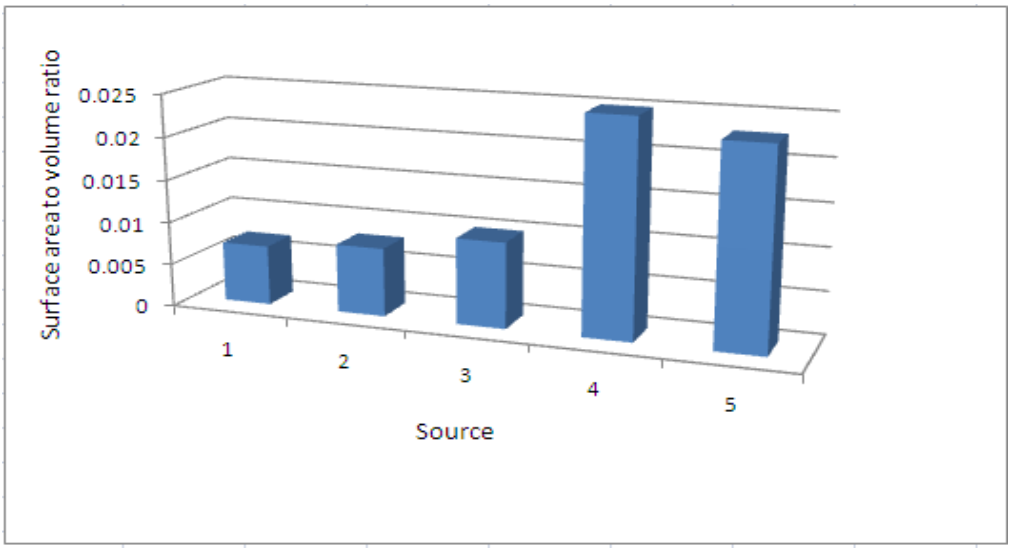

Figure 6 a : Surface-area-to-volume-ratio of BSA nanoparticles from different sources at the targeted size of 100nm

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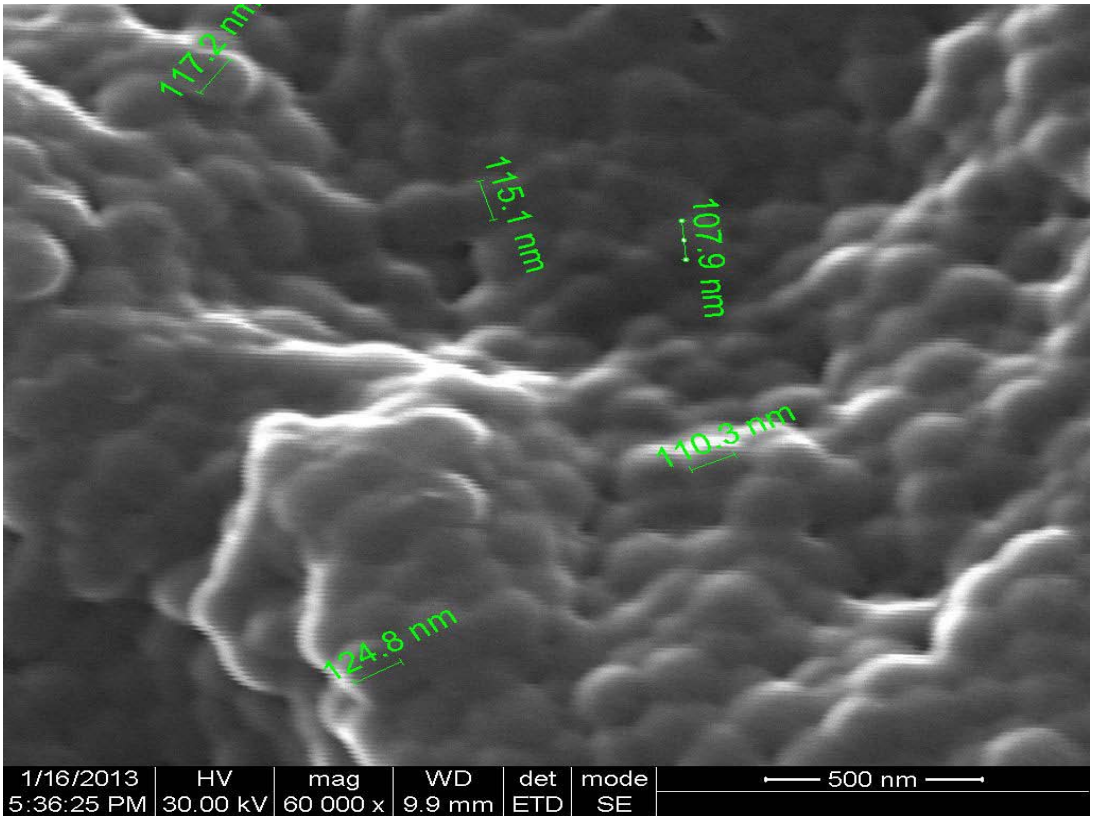

Figure 7 : Scanning electron micrographs of BSA nanoparticles at the targeted size of 100nm by Intermittent Method.

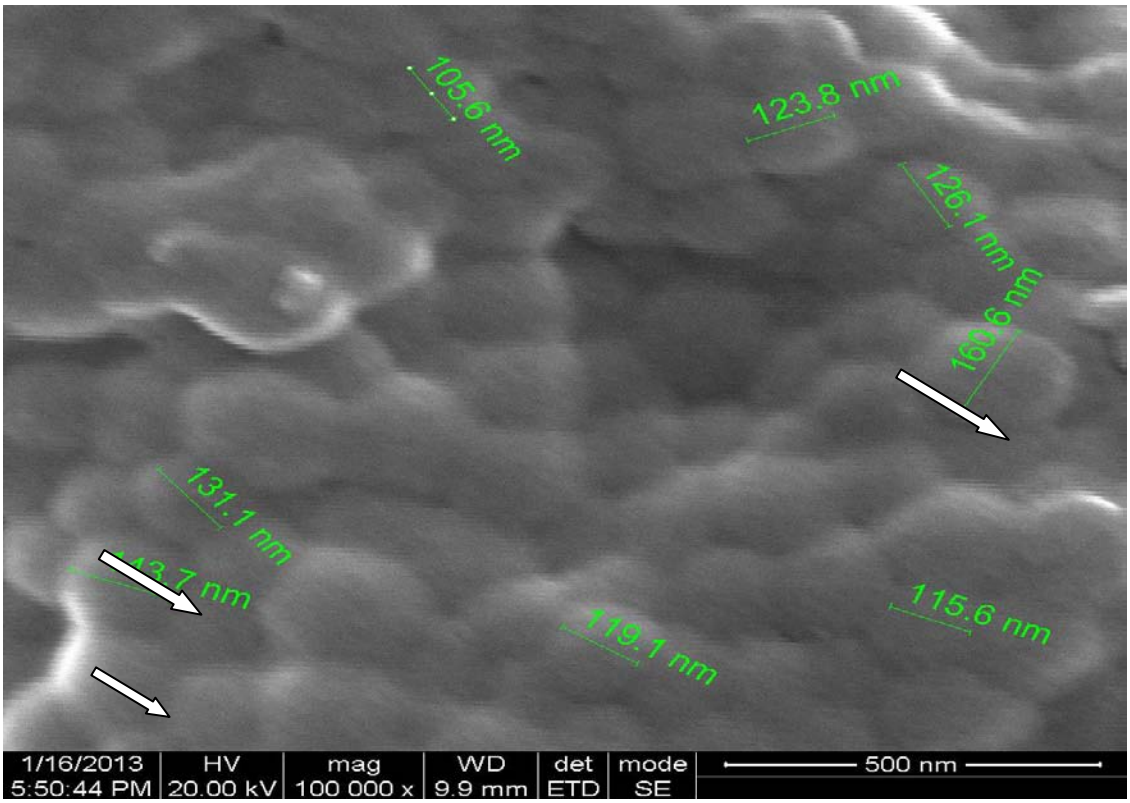

Figure 8 : Scanning electron micrograph of BSA nanoparticles at the targeted size of 100nm by Continuous Method

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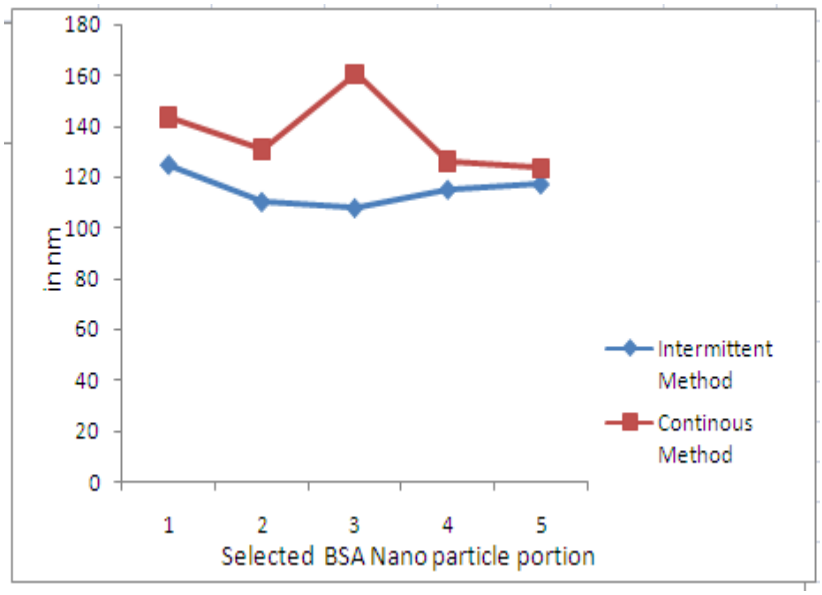

Figure 8 a : The Size of BSA Nanoparticles by the Intermittent and Continuous Method

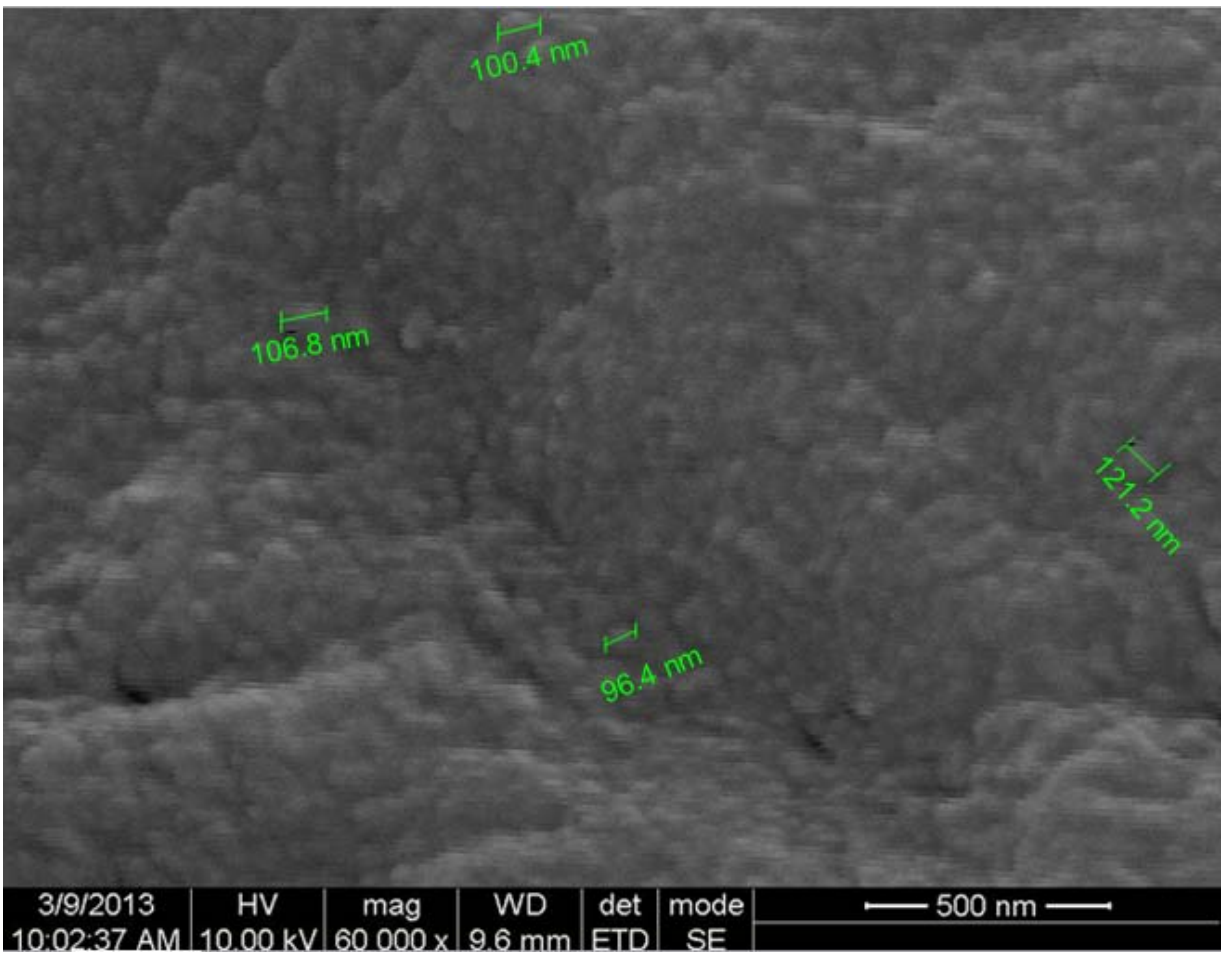

Figure 9 : Scanning electron micrographs of BSA nanoparticle encapsulated enzyme (Enz-A) at the targeted size of 100nm by Intermittent Method.

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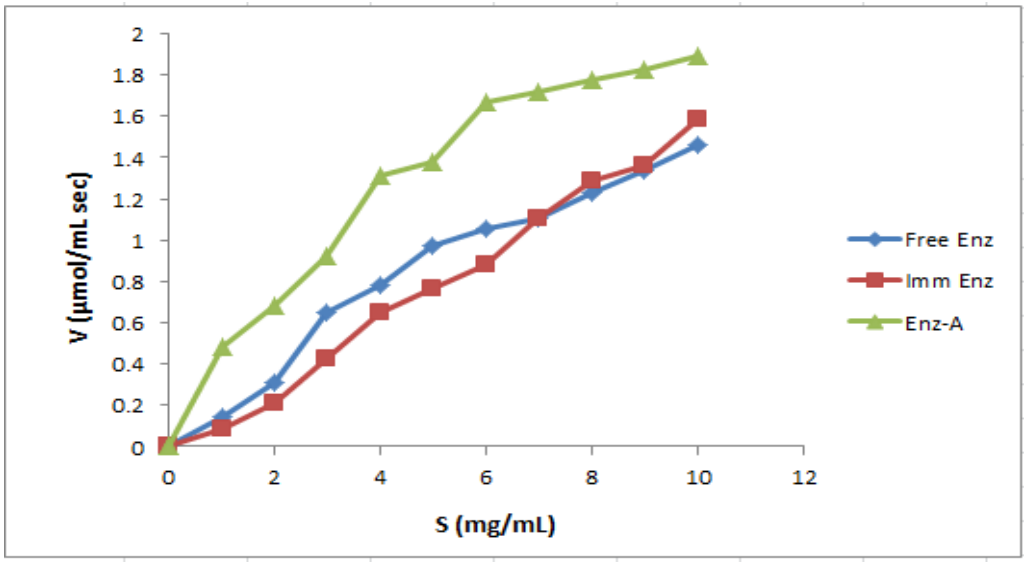

Figure 10 : Kinetic analysis of Enz-A with free and immobilised α-amylase enzyme

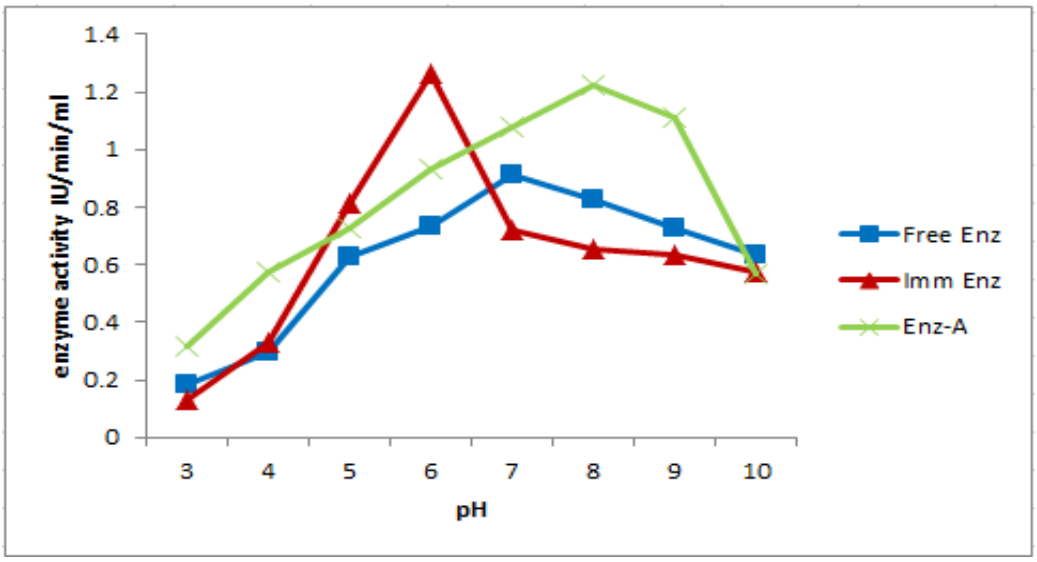

Figure 11 : Effect of pH on activity of Enz-A, Free and Immobilized α-amylase

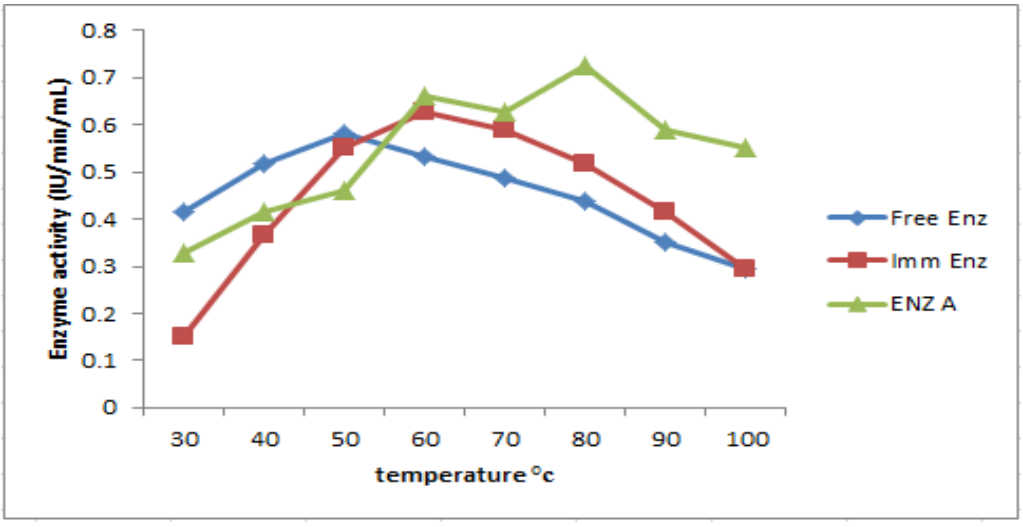

Figure 12 : Effect of temperature on activity of Enz-A, Free and Immobilized α- amylase enzyme

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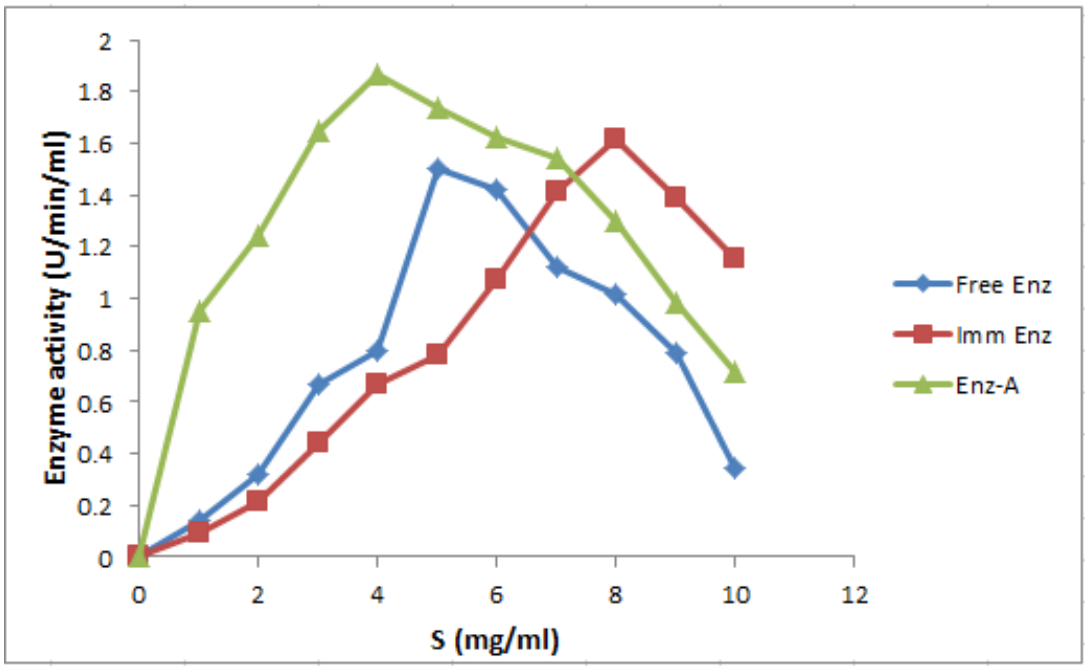

Figure 13 : Effect of substrate on activity of Enz-A, Free and Immobilized α- amylase enzyme

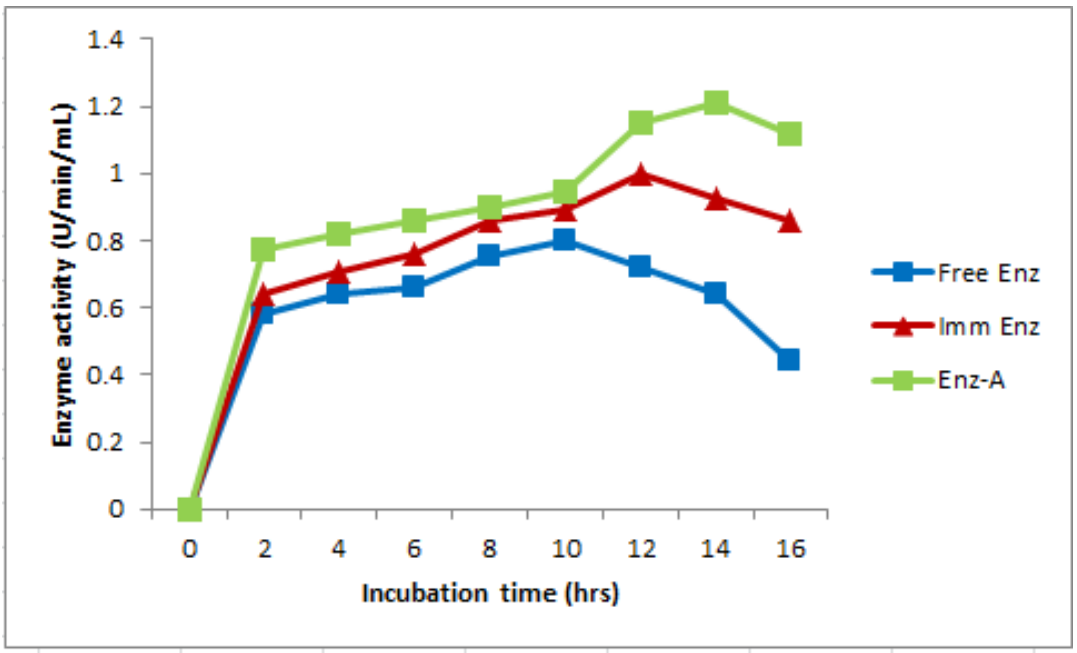

Figure 14 : Effect of incubation time on activity of Enz-A, Free and Immobilized α- Amylase enzyme

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Review papers: These are concise, significant but helpful and decisive topics for young researchers.

Research articles: These are handled with small investigation and applications

Research letters: The letters are small and concise comments on previously published matters.

5.STRUCTURE AND FORMAT OF MANUSCRIPT

The recommended size of original research paper is less than seven thousand words, review papers fewer than seven thousands words also.Preparation of research paper or how to write research paper, are major hurdle, while writing manuscript. The research articles and research letters should be fewer than three thousand words, the structure original research paper; sometime review paper should be as follows:

Papers: These are reports of significant research (typically less than 7000 words equivalent, including tables, figures, references), and comprise:

(a)Title should be relevant and commensurate with the theme of the paper.

(b) A brief Summary, "Abstract" (less than 150 words) containing the major results and conclusions.

(c) Up to ten keywords, that precisely identifies the paper's subject, purpose, and focus.

(d) An Introduction, giving necessary background excluding subheadings; objectives must be clearly declared.

(e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition; sources of information must be given and numerical methods must be specified by reference, unless non-standard.

(f) Results should be presented concisely, by well-designed tables and/or figures; the same data may not be used in both; suitable statistical data should be given. All data must be obtained with attention to numerical detail in the planning stage. As reproduced design has been recognized to be important to experiments for a considerable time, the Editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned un-refereed;

(g) Discussion should cover the implications and consequences, not just recapitulating the results; conclusions should be summarizing.

(h) Brief Acknowledgements.

(i) References in the proper form.

Authors should very cautiously consider the preparation of papers to ensure that they communicate efficiently. Papers are much more likely to be accepted, if they are cautiously designed and laid out, contain few or no errors, are summarizing, and be conventional to the approach and instructions. They will in addition, be published with much less delays than those that require much technical and editorial correction.

The Editorial Board reserves the right to make literary corrections and to make suggestions to improve briefness.

It is vital, that authors take care in submitting a manuscript that is written in simple language and adheres to published guidelines.

Format

Language: The language of publication is UK English. Authors, for whom English is a second language, must have their manuscript efficiently edited by an English-speaking person before submission to make sure that, the English is of high excellence. It is preferable, that manuscripts should be professionally edited.

Standard Usage, Abbreviations, and Units: Spelling and hyphenation should be conventional to The Concise Oxford English Dictionary. Statistics and measurements should at all times be given in figures, e.g. 16 min, except for when the number begins a sentence. When the number does not refer to a unit of measurement it should be spelt in full unless, it is 160 or greater.

Abbreviations supposed to be used carefully. The abbreviated name or expression is supposed to be cited in full at first usage, followed by the conventional abbreviation in parentheses.

Metric SI units are supposed to generally be used excluding where they conflict with current practice or are confusing. For illustration, 1.4 l rather than 1.4 × 10-3 m3, or 4 mm somewhat than 4 × 10-3 m. Chemical formula and solutions must identify the form used, e.g. anhydrous or hydrated, and the concentration must be in clearly defined units. Common species names should be followed by underlines at the first mention. For following use the generic name should be constricted to a single letter, if it is clear.

Structure

All manuscripts submitted to Global Journals Inc. (US), ought to include:

Title: The title page must carry an instructive title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) wherever the work was carried out. The full postal address in addition with the e-mail address of related author must be given. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining and indexing.

Abstract, used in Original Papers and Reviews:

Optimizing Abstract for Search Engines

Many researchers searching for information online will use search engines such as Google, Yahoo or similar. By optimizing your paper for search engines, you will amplify the chance of someone finding it. This in turn will make it more likely to be viewed and/or cited in a further work. Global Journals Inc. (US) have compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

Key Words

A major linchpin in research work for the writing research paper is the keyword search, which one will employ to find both library and Internet resources.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy and planning a list of possible keywords and phrases to try.

Search engines for most searches, use Boolean searching, which is somewhat different from Internet searches. The Boolean search uses "operators," words (and, or, not, and near) that enable you to expand or narrow your affords. Tips for research paper while preparing research paper are very helpful guideline of research paper.

Choice of key words is first tool of tips to write research paper. Research paper writing is an art.A few tips for deciding as strategically as possible about keyword search:

- One should start brainstorming lists of possible keywords before even begin searching. Think about the most important concepts related to research work. Ask, "What words would a source have to include to be truly valuable in research paper?" Then consider synonyms for the important words.
- It may take the discovery of only one relevant paper to let steer in the right keyword direction because in most databases, the keywords under which a research paper is abstracted are listed with the paper.
- One should avoid outdated words.

Keywords are the key that opens a door to research work sources. Keyword searching is an art in which researcher's skills are bound to improve with experience and time.

Numerical Methods: Numerical methods used should be clear and, where appropriate, supported by references.

Acknowledgements: Please make these as concise as possible.

References

References follow the Harvard scheme of referencing. References in the text should cite the authors' names followed by the time of their publication, unless there are three or more authors when simply the first author's name is quoted followed by et al. unpublished work has to only be cited where necessary, and only in the text. Copies of references in press in other journals have to be supplied with submitted typescripts. It is necessary that all citations and references be carefully checked before submission, as mistakes or omissions will cause delays.

References to information on the World Wide Web can be given, but only if the information is available without charge to readers on an official site. Wikipedia and Similar websites are not allowed where anyone can change the information. Authors will be asked to make available electronic copies of the cited information for inclusion on the Global Journals Inc. (US) homepage at the judgment of the Editorial Board.

The Editorial Board and Global Journals Inc. (US) recommend that, citation of online-published papers and other material should be done via a DOI (digital object identifier). If an author cites anything, which does not have a DOI, they run the risk of the cited material not being noticeable.

The Editorial Board and Global Journals Inc. (US) recommend the use of a tool such as Reference Manager for reference management and formatting.

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Tables: Tables should be few in number, cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g. Table 4, a self-explanatory caption and be on a separate sheet. Vertical lines should not be used.

Figures: Figures are supposed to be submitted as separate files. Always take in a citation in the text for each figure using Arabic numbers, e.g. Fig. 4. Artwork must be submitted online in electronic form by e-mailing them.

Preparation of Electronic Figures for Publication

Even though low quality images are sufficient for review purposes, print publication requires high quality images to prevent the final product being blurred or fuzzy. Submit (or e-mail) EPS (line art) or TIFF (halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Do not use pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings) in relation to the imitation size. Please give the data for figures in black and white or submit a Color Work Agreement Form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution (at final image size) ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs) : >350 dpi; figures containing both halftone and line images: >650 dpi.

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Upon approval of a paper for publication, the manuscript will be forwarded to the dean, who is responsible for the publication of the Global Journals Inc. (US).

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(Free of charge) from the following website:

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TECHNIQUES FOR WRITING A GOOD QUALITY RESEARCH PAPER:

1. Choosing the topic: In most cases, the topic is searched by the interest of author but it can be also suggested by the guides. You can have several topics and then you can judge that in which topic or subject you are finding yourself most comfortable. This can be done by asking several questions to yourself, like Will I be able to carry our search in this area? Will I find all necessary recourses to accomplish the search? Will I be able to find all information in this field area? If the answer of these types of questions will be "Yes" then you can choose that topic. In most of the cases, you may have to conduct the surveys and have to visit several places because this field is related to Computer Science and Information Technology. Also, you may have to do a lot of work to find all rise and falls regarding the various data of that subject. Sometimes, detailed information plays a vital role, instead of short information.

2. Evaluators are human: First thing to remember that evaluators are also human being. They are not only meant for rejecting a paper. They are here to evaluate your paper. So, present your Best.

3. Think Like Evaluators: If you are in a confusion or getting demotivated that your paper will be accepted by evaluators or not, then think and try to evaluate your paper like an Evaluator. Try to understand that what an evaluator wants in your research paper and automatically you will have your answer.

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7. Use right software: Always use good quality software packages. If you are not capable to judge good software then you can lose quality of your paper unknowingly. There are various software programs available to help you, which you can get through Internet.

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9. Use and get big pictures: Always use encyclopedias, Wikipedia to get pictures so that you can go into the depth.

10. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right! It is a good habit, which helps to not to lose your continuity. You should always use bookmarks while searching on Internet also, which will make your search easier.

11. Revise what you wrote: When you write anything, always read it, summarize it and then finalize it.

12. Make all efforts: Make all efforts to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in introduction, that what is the need of a particular research paper. Polish your work by good skill of writing and always give an evaluator, what he wants.

13. Have backups: When you are going to do any important thing like making research paper, you should always have backup copies of it either in your computer or in paper. This will help you to not to lose any of your important.

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15. Use of direct quotes: When you do research relevant to literature, history or current affairs then use of quotes become essential but if study is relevant to science then use of quotes is not preferable.

16. Use proper verb tense: Use proper verb tenses in your paper. Use past tense, to present those events that happened. Use present tense to indicate events that are going on. Use future tense to indicate future happening events. Use of improper and wrong tenses will confuse the evaluator. Avoid the sentences that are incomplete.

17. Never use online paper: If you are getting any paper on Internet, then never use it as your research paper because it might be possible that evaluator has already seen it or maybe it is outdated version.

18. Pick a good study spot: To do your research studies always try to pick a spot, which is quiet. Every spot is not for studies. Spot that suits you choose it and proceed further.

19. Know what you know: Always try to know, what you know by making objectives. Else, you will be confused and cannot achieve your target.

20. Use good quality grammar: Always use a good quality grammar and use words that will throw positive impact on evaluator. Use of good quality grammar does not mean to use tough words, that for each word the evaluator has to go through dictionary. Do not start sentence with a conjunction. Do not fragment sentences. Eliminate one-word sentences. Ignore passive voice. Do not ever use a big word when a diminutive one would suffice. Verbs have to be in agreement with their subjects. Prepositions are not expressions to finish sentences with. It is incorrect to ever divide an infinitive. Avoid clichés like the disease. Also, always shun irritating alliteration. Use language that is simple and straight forward. put together a neat summary.

21. Arrangement of information: Each section of the main body should start with an opening sentence and there should be a changeover at the end of the section. Give only valid and powerful arguments to your topic. You may also maintain your arguments with records.

22. Never start in last minute: Always start at right time and give enough time to research work. Leaving everything to the last minute will degrade your paper and spoil your work.

23. Multitasking in research is not good: Doing several things at the same time proves bad habit in case of research activity. Research is an area, where everything has a particular time slot. Divide your research work in parts and do particular part in particular time slot.

24. Never copy others' work: Never copy others' work and give it your name because if evaluator has seen it anywhere you will be in trouble.

25. Take proper rest and food: No matter how many hours you spend for your research activity, if you are not taking care of your health then all your efforts will be in vain. For a quality research, study is must, and this can be done by taking proper rest and food.

26. Go for seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

27. Refresh your mind after intervals: Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

28. Make colleagues: Always try to make colleagues. No matter how sharper or intelligent you are, if you make colleagues you can have several ideas, which will be helpful for your research.

29. Think technically: Always think technically. If anything happens, then search its reasons, its benefits, and demerits.

30. Think and then print: When you will go to print your paper, notice that tables are not be split, headings are not detached from their descriptions, and page sequence is maintained.

31. Adding unnecessary information: Do not add unnecessary information, like, I have used MS Excel to draw graph. Do not add irrelevant and inappropriate material. These all will create superfluous. Foreign terminology and phrases are not apropos. One should NEVER take a broad view. Analogy in script is like feathers on a snake. Not at all use a large word when a very small one would be sufficient. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Amplification is a billion times of inferior quality than sarcasm.

32. Never oversimplify everything: To add material in your research paper, never go for oversimplification. This will definitely irritate the evaluator. Be more or less specific. Also too, by no means, ever use rhythmic redundancies. Contractions aren't essential and shouldn't be there used. Comparisons are as terrible as clichés. Give up ampersands and abbreviations, and so on. Remove commas, that are, not necessary. Parenthetical words however should be together with this in commas. Understatement is all the time the complete best way to put onward earth-shaking thoughts. Give a detailed literary review.

33. Report concluded results: Use concluded results. From raw data, filter the results and then conclude your studies based on measurements and observations taken. Significant figures and appropriate number of decimal places should be used. Parenthetical remarks are prohibitive. Proofread carefully at final stage. In the end give outline to your arguments. Spot out perspectives of further study of this subject. Justify your conclusion by at the bottom of them with sufficient justifications and examples.

34. After conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print to the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects in your research.

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Key points to remember:

- Submit all work in its final form.
- Write your paper in the form, which is presented in the guidelines using the template.
- Please note the criterion for grading the final paper by peer-reviewers.

Final Points:

A purpose of organizing a research paper is to let people to interpret your effort selectively. The journal requires the following sections, submitted in the order listed, each section to start on a new page.

The introduction will be compiled from reference matter and will reflect the design processes or outline of basis that direct you to make study. As you will carry out the process of study, the method and process section will be constructed as like that. The result segment will show related statistics in nearly sequential order and will direct the reviewers next to the similar intellectual paths throughout the data that you took to carry out your study. The discussion section will provide understanding of the data and projections as to the implication of the results. The use of good quality references all through the paper will give the effort trustworthiness by representing an alertness of prior workings.

Writing a research paper is not an easy job no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record keeping are the only means to make straightforward the progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear

· Adhere to recommended page limits

Mistakes to evade

- Insertion a title at the foot of a page with the subsequent text on the next page
- Separating a table/chart or figure - impound each figure/table to a single page
- Submitting a manuscript with pages out of sequence

In every sections of your document

· Use standard writing style including articles ("a", "the," etc.)

· Keep on paying attention on the research topic of the paper

· Use paragraphs to split each significant point (excluding for the abstract)

· Align the primary line of each section

· Present your points in sound order

· Use present tense to report well accepted

· Use past tense to describe specific results

· Shun familiar wording, don't address the reviewer directly, and don't use slang, slang language, or superlatives

· Shun use of extra pictures - include only those figures essential to presenting results

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Choose a revealing title. It should be short. It should not have non-standard acronyms or abbreviations. It should not exceed two printed lines. It should include the name(s) and address (es) of all authors.

Abstract:

The summary should be two hundred words or less. It should briefly and clearly explain the key findings reported in the manuscript--must have precise statistics. It should not have abnormal acronyms or abbreviations. It should be logical in itself. Shun citing references at this point.

An abstract is a brief distinct paragraph summary of finished work or work in development. In a minute or less a reviewer can be taught the foundation behind the study, common approach to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Yet, use comprehensive sentences and do not let go readability for briefness. You can maintain it succinct by phrasing sentences so that they provide more than lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study, with the subsequent elements in any summary. Try to maintain the initial two items to no more than one ruling each.

- Reason of the study - theory, overall issue, purpose
- Fundamental goal
- To the point depiction of the research
- Consequences, including definite statistics - if the consequences are quantitative in nature, account quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
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- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
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Introduction:

The **Introduction** should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

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Materials:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

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The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.

Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

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- Make a decision if each premise is supported, discarded, or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."
- Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work
- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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