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Conversion of Corn Waste into Value Added Products

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CONVERSION OF CORN WASTE INTO VALUE ADDED PRODUCTS

A thesis submitted

in partial fulfillment for the Degree of

Doctor of Philosophy

by

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(Under the supervision of Professor Dr. Ibrahim H. Mondal and Professor Dr. Md. Abu Sayeed)

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FEBRUARY, 2016

DEDICATED
TO
MY SON, MOTHER
AND
UNCLE

DECLARATION

I hereby declare that the thesis work submitted as entitled **"Conversion of Corn Waste into Value Added Products"** under the supervision of Professor Dr. Md. Ibrahim H. Mondal and Professor Dr. Md. Abu Sayeed in the Department of Applied Chemistry and Chemical Engineering, University of Rajshahi, Rajshahi-6205, Bangladesh for the assessment on the programme of study leading to the award of Doctor of Philosophy (Ph.D.) is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not breach any law of copyright, and has not been taken from the work of others save except references used in the text of the thesis.

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CERTIFICATE

This is to certify that the thesis entitled **"Conversion of Corn Waste into Value Added Products"** submitted by **Mst Sarmina Yeasmin,** Roll No.: 11105, Registration No.: 2723, Session: 2011-2012, to the Institute of Environmental Science (IES), University of Rajshahi, Bangladesh, in partial fulfillment for the award of the degree of **Doctor of Philosophy** is a *bonafide* record of research work carried out by her under our supervision. To the best of our knowledge, the contents of this thesis, in full or in parts, have not been submitted to any other Institution or University for the award of any degree or diploma.

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CONVERSION OF CORN WASTE INTO VALUE ADDED PRODUCTS

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ABSTRACT

Corn waste is an abundant lignocellulosic agricultural waste. The chemical composition of corn waste such as stalks, husks, cobs and leaves was analyzed to get cellulose, hemicelluloses, lignin, fatty and waxy matters, pectic matters and aqueous extract. Corn husk contained higher amount of cellulose (45%), was furnished as the raw material for the production of food-grade carboxymethyl cellulose (CMC). Cellulose was carboxymethylated using sodium hydroxide (NaOH) and monochloroacetic acid (MCA), in aqueous ethanolic medium, under heterogeneous conditions. The carboxymethylation reaction was optimized in terms of DS with respect to the cellulose particle size, NaOH concentration, MCA concentration, reaction temperature and reaction time. By-products of the etherification reaction such as sodium glycolate and sodium chloride were removed in a series of alcohol washes and separations, and sodium salts content in the purified CMC was determined. The degree of substitution (DS) was determined using chemical method. The CMC product had an optimized DS of 2.41 and the optimal conditions for carboxymethylation were NaOH concentration, 7.5 $molL^{-1}$; MCA concentration, 12 $molL^{-1}$; reaction temperature, 55°C; reaction time, 3.5 h and cellulose particle size, 74 µm. These optimization factors allowed to prepare highly substituted CMC with higher purity, providing plenty of opportunities for its manifold applications.

Qualitative and quantitative analyses of CMC were carried out to determine molecular weight, purity, yield, solubility; water and oil holding capacities, moisture, ash and gel contents. Characterizations of cellulose and CMC were carried out by analyzing the spectra of FTIR, XRD patterns, SEM photomicrographs and TGA thermogram. The molecular structure of the CMC was determined by the analysis of ^{13}C NMR spectra. Microbiological testing of the prepared CMC was done by the pour plate method. Concentrations of heavy metals in the purified CMC were measured by AAS technique and found to be within the WHO/FAO recommended value. Solubility, yield, molecular weight and DS of CMC increased with decreased cellulose particle sizes. A comparative study with CMC available in the

international market was conducted. The purity of the prepared CMC was higher, at 99.99% well above the purity of 99.5% for standard CMC. High purity CMC showed yield 2.4 g/g , water holding capacity 5.11 g/g, oil holding capacity 1.59 g/g, ash content 18.05%, moisture content 2.21% & gel content 99.96%.

Extensive analysis for potential external contaminants of CMC such as heavy metals (arsenic, lead, cadmium, mercury) and microbial contaminants, such as Total plate count, Yeast and Mold, *Colliform, Salmonella* and *E. coli* that are generally associated with food products, suggest either the absence of these contaminants or their presence at very low levels that are considered as safe. Similarly, the presence of processing aids and by-products from the manufacturing are minimized in the final product to levels that are safe for human consumption. The product meets or exceeds the specification or purity requirements of the FCC, USP and JECFA.

A study was conducted to assess the toxicity of prepared CMC when administered orally, via dietary admixture, to Swiss albino mice (5/sex/group) at dose levels of 0 (control), 5, 10, and 20 mg/g body weight/day for a period of at least 3 months. Studies were conducted in compliance with OECD principles of Good Laboratory Practice and according to OECD Guidelines 408. Animals were observed for mortality, morbidity, body weight changes, feed and water intake. Urinary, biochemical and haematological assessments as well as body and organ weights of the mice were carried out at every one month. After 3 months of treatment, all mice scheduled for terminal sacrifice were killed and selected organs were weighed. Complete macroscopic examinations and histopathological evaluation of selected tissues were conducted on all animals.

No major abnormalities in the histopathology of liver, kidney, heart and lung were detected compared to control group under microscope. This indicated that the compound has no toxic effect on cellular structure. No mortality occurred during the study. Water intake, urine production and urinary sodium, calcium and citrate excretion increased with increasing doses of CMC due to their increasing sodium intake. Body weight, water and food consumption, haematology, clinical biochemistry, behavioural observational assessments and organ weights did not reveal any significant, consistent, dose-dependent test article-related adverse effects. A no observed adverse effect level (NOAEL) from the present study was determined to be 20 mg/g body weight/day for mice under the conditions of this study, the highest dose tested. These findings support the safety of CMC for use as an excipient or as food additives and for pharmaceuticals.

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LIST OF ABBREVIATIONS

A	Mark-Houwink-Sakurada exponent
AACC	American Association for Clinical Chemistry
ADI	Acceptable daily intake
ADP	Adenosine diphosphate
AGU	Anhydroglucose units
ALT	Alanine aminotransferase
AMP	2-amino-2-methyl-1-propanol
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ASTM	American Society for Testing and Materials
ATP	Adenosine triphosphate
BBS	Bangladesh Bureau of Statistics
CFR	Code of Federal Regulations
Cfu	Colony-forming unit
CHE	Cholesterol esterase
CHOD	Cholesterol oxidase
Cm	Centimeter
CMC	Carboxymethyl cellulose
D.P.X.	Distyrene, plasticizer and xylene
D_2O	Deuterium oxide
Da	Dalton
DC	Differential count
DS	Degree of substitution
DTG	Derivative thermogravimetry
e.g.	Exempli gratia (For example)
EDTA	Ethylene diamine tetraacetic acid
EFSA	European Food Safety Authority
EMB	Eosin Methylene Blue
ESPA	N-Ethyl-N-(3-sulfopropyl)-m-anisidine
et al.	et alii/alia (and others)

Etc	et cetera
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FTIR	Fourier transform infrared
G	Gram
GK	Glycerol kinase
GLDH	Glutamate dehydrogenase
GOD-PAP	Glucose-Oxidase
GPO	Glycerol phosphate oxidase
GRAS	Generally recognized as safe
h	Hour
i.e	id est (that is)
IAEC	Institutional Animal Ethical Committee
IFCC	International Federation of Clinical Chemistry
IU/L	International units per litre
JECFA	Joint Expert Committee on Food Additives
kV	Kilovolt
lb/sq.in	Pound per square inch
LDH	Lactic dehydrogenase
Linn or L.	Linnaeus
mA	Milliampere
MDH	Malate dehydrogenase
mEq/L	Milliequivalents per litre
mg	Milligram
mg/dL	Milligrams per deciliter
MHz	Megahertz
min	Minute
ml	Milliliter
mmol/L	Milli mol per litre
mol/L	Mol per litre
mt/ha	Metric ton per hectare
N	Normality
NAD^+	Oxidized form of nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen

NMR	Nuclear magnetic resonance
NOAEL	No- observed- adverse- effect- level
OECD	Organization for Economic Co-operation and Development
OHC	Oil holding capacity
pH	Anti logarithms of hydrogen ion concentration
pNP	p-nitrophenol
pNPP	p-nitrophenyl phosphate
POD	Peroxidase
ppm	Parts per million
RBC	Red blood cells
rpm	Rotation per minute
SALP	Serum alkaline phosphatase
SCF	Scientific Committee on Food
SCP	Single cell protein
SEM	Scanning electron microscope
SEM	Standard error of mean
SGOT	Serum-glutamate-oxaloacetate-transaminase
SGPT	Serum-glutamate-pyruvate-transaminase
TAPPI	Technical association of the pulp and paper industry
TC	Total count
TGA	Thermogravimetric analysis
U/L	Units per litre
USP	United States Pharmacopeia
UV	Ultraviolet
WBC	White blood cells
WHC	Water holding capacity
WHO	World Health Organization
WVTR	Water vapor transmission rate
µm	Micrometer
4-AAP	4-aminoantipyrine
4-AP	4-aminophenazone

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

There has been increasing interest in cellulose based-materials due to the abundance, renewable and eco-friendly nature of cellulose [Hubbe *et al.,* 2008]. Modified cellulose is now advancing in terms of productions and innovations due to the available resource of natural polymer in the world. The resources can range from woods to even the agricultural waste.

Due to strong inter- and intra-molecular hydrogen bonds, cellulose neither melts nor dissolves readily in hot or cold water [Choi *et al.*, 2007] and in most common organic solvents [Hattori *et al.*, 2004; Adinugraha *et al.,* 2005]. In order to utilize cellulose in the food industry, cellulose must be converted into its derivatives. One of the most common derivatives is carboxymethyl cellulose (CMC), also referred to as Na-CMC.

Recently CMC has got ample scientific attention, especially due to its polyelectrolyte character and it is the most widely used cellulose ether today with applications in many areas of industry and human life [Methacanon *et al.*, 2003] with different grade. CMC of different grades are mainly signified by the DS. However, a literature search found no published reports of producing a higher DS at single-step carboxymethylation.

High purity grades are employed as food additives [Mohkami and Talaeipour, 2011] also known as cellulose gum [Barba *et al.*, 2002].

Some papers have reported the synthesis of CMC from various cellulosic sources [Heydarzadeh *et al.,* 2009; He *et al.*, 2009; Egbuchunam and Okicimen, 2003; Nagieb *et al.*, 2001; Jahan *et al.*, 2007; Gu *et al.*, 2012]. There is considerable interest in finding cheaper alternatives for producing CMC.

Every year in Bangladesh, large amount of CMC is being imported to meets her demand and the importance of CMC is increasing day by day [Mondal and Alam, 2013]. Therefore, there is a need to produce CMC from locally available cheap raw material, such as agricultural waste, economically on a large scale.

Corn is one of the most important cereal crops grown widely throughout the world. Recently cultivation of corn is tremendously increased in Bangladesh [Uddin *et al.*, 2013] to meet her growing demand and huge amount of corn residue are thrown away as waste. The utilization of these wastes from agricultural farm products can be of immense importance. Many researchers have tried to transform this waste into valuable products. However, very little has been published on the utilization of corn waste.

This chapter introduces a background about corn production, corn waste and its utiization, lignocellulosic materials and its composition, with an overview of environmental concerns. This chapter also reviews detail about CMC with its structure, production, characteristics, applications, and about agricultural source of preparation. The chapter also elaborates safety evaluations of CMC.

1.2 Present status of corn

Corn also known as maize (*Zea mays* L.) is an amazing crop throughout the world which occupies a large portion of world economy. Total corn production in the world for the 2013-14 is 959 million tons which is more than wheat (709 million tons) and rice (473 million tons) [GMR, 2014]. In 2009, over 159 million hectares of corn were planted worldwide which gave a yield of 5.12 tons/ha [FAOSTAT, 2009]. From nutritional view point it is better than rice and wheat [http://ndb.nal.usda.gov/ndb/search/list]. The global average yield of corn is more than 4 tons per hectare [Paliwal, 2000b; Farnham *et al.,* 2003].

In Bangladesh, corn commonly named as vutta is considered as second most important cereal crop which occupies 165.5 thousand hectares land and produces 1018 thousand metric tons with an average yield of 6.1 tons/ha [BBS, 2011]. Approximately 1,850,656 metric tons of corn is grown per year in Bangladesh [Food and Agri. Stat. of UN, 2012] and current need of corn is 1.60 million metric tons annually [Ahmed, 2013].

The area, production and yield of wheat and corn from 1999-00 to 2005-06 are presented in **Table 1.1**. It can be revealed from the table that area, production, and yield of wheat are decreasing in each year from 1999-00 to 2005-06, while it is increasing for corn in each year during the same period. Its position is 1st among the cereals in terms of yield (corn, 5.30 mt/ha; wheat, 1.60 mt/ha and rice, 2.52 mt/ha], but in terms of area and production, it ranks 3rd just after rice and wheat [BBS, 2006]. According to the Bangladesh Bureau of Statistics, corn cultivation areas in the country jumped from 9% to 15% in 2010 to 2011 [Moniruzzaman *et al.,* 2009].

Demand for corn is increasing day by day in the world as well as in Bangladesh due to its diversified uses. Because, it is a high yielding and low-cost crop compared to rice and wheat. Its tolerance to variation in weather conditions is also making farmers switching from wheat to corn in the winter.

Table 1.1 Area, production and yield of wheat and corn over the period from 1999-2000 to 2005-2006.

Year	Wheat			Corn		
	Area [ha]	Production [mt]	Yield [mt/ha]	Area [ha]	Production [mt]	Yield [mt/ha]
1999-00	832000	1840000	2.21	3161	4075	1.29
2000-01	772000	1673000	2.17	4901	10350	2.11
2001-02	742000	1606000	2.16	19972	64335	3.22
2002-03	706000	1507000	2.13	29059	117255	4.04
2003-04	567000	1248000	2.20	50030	241460	4.83
2004-05	558000	976000	1.75	66803	356280	5.33
2005-06	481000	772000	1.60	98408	521525	5.30

Source: BBS [2003, 2006]

1.3 Corn wastes

Waste can be defined as something useless produced by the same action that produces something useful or a by-product of industrial, agricultural municipal and mining activities [Aderemi *et al.*, 2011]. The by-products of agricultural activities are usually referred to as "agricultural waste" because they are not the primary products. These wastes chiefly take the form of crop residues (residual stalks, straw, leaves, roots, husks, shells etc.). Agricultural wastes are widely available, renewable and virtually free, hence they can be an important resource [Sabiiti *et al.*, 2005].

The wastes of corn which are left behind after harvest include the husks, cobs, chaff, stalks and the leaves. Husks, cobs and stalks are among the prominent waste associated with corn [Oseni and Ekperigin, 2007]. Annually approximately 204 million dry metric tons of corn residue are returned to the ground as waste by-product in corn grain production [Perlack *et al.,* 2005]. It is reported by Sokhansanj *et al.*, [2010] that for every 1 kg of dry corn grains, about 0.15 kg of cobs, 0.22 kg of leaves, 0.41 kg of husks and 0.50 kg of stalks are produced.

Approximately 50 percent of the weight of the total corn plant is residue, consisting of stalk, leaf, cob and husk [https://www.ag. ndsu.edu/pubs /ansci/ beef/ as1548. pdf]. **Table I.2** indicates the dry matter distribution in corn residue at or immediately following corn harvest. The stalk, cob, husk all contain more than one-half of the dry matter weight of the total residue material.

Since, in the recent years, the cultivation of corn has been increased tremendously in Bangladesh and the yield is also very satisfactory, so waste from corn processing area is also increasing day by day. Every year after harvesting and separating the corn kernels, a large amount of corn wastes such as cob, hull, stalk, husk, leaves etc, remain unused. Sometimes they are used in less important purposes; mostly they are thrown away as wastes which are the causes of environment pollution.

Waste represents the loss of both material and energy resources [Marmo, 2003]. For a sustainable development of society, resources have to be recycled as much as possible. With the increasing demand for people and livestock products in the world economy and shrinking land area, future hope of their food security will largely depend on the better utilization of their non-conventional feed resources

[Makkar, 2000]. So, comprehensible plan is needed to make the corn residue sustainable.

Table 1.2 Dry matter distribution in corn residues.

Corn Residues	% Moisture	% of Residue D.M. Basis
Stalk	70-75	31
Leaf	20-25	76
Cob	50-55	58
Husk	45-50	55

Adapted from 'Grazing Crop Residues', University of Nebraska publication EC 98-278-B.

1.4 Corn wastes and the environmental impact assessment

The impact of agricultural waste on the environment depends not only on the amounts generated but also on the disposal methods used. Some of the disposal practices pollute the environment [Sabiiti *et al.*, 2004; Tumuhairwe *et al.*, 2009].

Pollution crisis is a major environmental problem all around the world. It has adversely affected the lives of millions of people and caused many deaths and health disorders. Without pollution control or waste minimization, the waste products from agriculture and other activities, whether they accumulate or disperse, will degrade the environment. The environmental impacts of corn waste have been extensively studied thorough presentation and literature review.

The wastes of corn (straw), which is left after corn harvesting, sometimes left behind on the land or after eating the corn, the cobs form liters in the environment in the developing countries [Oseni and Ekperigin, 2007]. According to Van der Wal [1978], these cellulose-rich substrates form a total of more than 1,800 million tons

annually of renewable resources and the 95 percent residue needs considerable processing be it physical, chemical, or via some form of bioconversion before it can be turned into suitable feed or food.

Although wheat straw and rice straw are already being fully utilized by many farmers, either for bedding poultry farm (wheat straw in particular) or for feeding livestock, yet corn stalks, corn cobs and husks are not being utilized on large scale [Hathout and EL-Nouby, 1990]. Most of these compounds are difficult to decompose rapidly in the natural environment [Knauf and Moniruzzaman, 2004; Reddy and Young, 2005].

Corn cultivation and processing area leaves considerable amounts of corn residues or wastes. Some of it is recycled into the agricultural production as fertilizer, some are utilized as fuel, very less is utilized as cattle feed, some are burnt in the field, mostly thrown away as waste and in many instances pose disposal problems.

When these wastes are thrown in any place, they are rotten and produce bad odour and dust. Many micro-organisms work on waste and cause many harmful diseases. Rotten wastes alter soil health by changing the pH of the environment and by adding compounds that can be considered contaminants, and increase of nitrate levels in ground water, due to rapid mineralization and subsequent leaching of organic nitrogen [Shoemaker *et al.*, 1973].

Uncontrolled burning of waste in the fields is not only producing and creating global warming, it is also wasting useful energy. Agricultural waste burning is a common practice in the undeveloped countries, but it is a source of atmospheric pollution. According to Ezcurra *et al.* [2001], agricultural waste burning releases pollutants such as carbon monoxide, nitrous oxide, nitrogen dioxide and particles

(smoke carbon). These pollutants are accompanied by the formation of ozone and nitric acid [Hegg *et al.*, 1987], hence contributing to acid deposition [Lacaux *et al.*, 1992] thereby posing risk to human and ecological health.

Oseni and Ekperigin [2007] have reported that the disposed corn wastes have some negative effects on the environment by causing a nuisance and health risk. Accumulation of these wastes just like other agro-based wastes in non-farming areas like homes, markets, schools and colleges and offices etc, poses a serious environmental threat to human beings because of the offensive particulates, liquids or mists and army of flies that usually emanate from heaps of these wastes [Eze and Ojike, 2012].

If fields have greater than normal levels of downed ears or standing corn, the risk of acidosis or founder may increase [https://www.ag. ndsu.edu/pubs /ansci/ beef/as 1548. pdf]. Blacno-Canqi and Lal [2007] have reported that, depositing of corn stover in the corn fields can have negative environmental and soil quality repercussions, including decreases in soil organic carbon, depletion of plant nutrients, decreases in the soil's ability to retain plant nutrients and plant available water, and increases in water runoff and soil erosion.

More than two billion people use agricultural residues as the primary fuel for their cooking and heating needs, leading to significant health, economic and environmental consequences:

- Almost 2 million deaths each year are caused by breathing smoke from indoor cooking fires

- Respiratory infections are the leading cause of death of young children worldwide

Adequate management of residual wastes are therefore the major challenges to be tackled in future years if these impacts are to be avoided.

Bangladesh is the ninth most populous country and twelfth most densely populated countries in the world [https://en.wikipedia.org/wiki/Waste management in Bangladesh]. With this population growth, there is an increasing problem of waste management. Till to date little attention has been given on this vital issue in Bangladesh to overcome this problem. If it could pay good attention to handle this problem properly, waste would be less generous to open water source, ground water and the environment without causing huge monetary involvement.

With efficient collection and treatment systems, waste from agricultural production can be utilized as useful chemicals or by-products. Thus, the importance and necessity for the investigation of corn waste, need to be overemphasized both from the environmental as well as economical point of view.

Corn field

Corn processing area

Corn leaves

Corn stalks

Corn cobs

Corn husks

1.5 Characteristics of corn wastes

Corn wastes are agricultural by-products consist of major proportions of cellulose and hemicellulose, while the rest comprises lignin, nitrogenous compounds and ash. They are lignocellulosic materials [Hon, 1996]. Lignocellusic materials is a broad term that can be applied to a wide range of materials generally derived from trees to agricultural residues [Edward *et al.*, 1996; Ibrahim *et al.*, 2010].

Han and Ciegler [1982], and Theander and Aman [1984] reported that, lignocellulosic materials consists of cellulose, hemicellulose and lignin as main components, in the ratio 4:3:3. These residues also contain ash and low content protein. Chang [1987] mentioned that, lignocellulosic materials are complex insoluble molecules made up of aromatic building blocks resistant to break down. This complex consists of three main components; cellulose, hemicelluloses and lignin with varying proportions. Chahal [1991] mentioned that, most of crop residues contain 30-45 % cellulose, 16-27 % hemicelluloses, 3-13 % lignin and 3.6- 7.2 % crude protein.

Cellulose, hemicelluloses and lignin are present in varying amounts in the different parts of the plant and they are intimately associated to form the structural framework of the plant cell wall. The composition of lignocellulose depends on plant species, age and growth conditions. Distribution of cellulose, hemicelluloses and lignin varies significantly between different plants [Fengel and Wegener, 1984; Jørgensen *et al.*, 2007]. Lignocellulosic crop residue is a natural renewable resource, largely unused and abundantly available source of raw materials that can be converted into useful cellulose derivatives and energy [Klass, 1998; Cheng and Zhu, 2008].

As corn waste is lignocellulosic material contain higher amount of cellulose, therefore most attention today must be given to possible use of this waste into more useful and highly value added products.

Thus, if the corn wastes are properly treated, it will play an important role to meet up the cellulose related needs at the lowest cost to the society whilst minimizing the environmental and social impacts.

1.6 Utilization of corn wastes

Annually, great amounts of cellulose wastes, which could be measured in many billions of tons, are produced worldwide as residues from agricultural activities [Petre *et al.*, 1999]. Hathout and El- Nouby [1990] found that, only 4.0 to 4.3 million tons of crop residues are used for feeding ruminants. The problem of feeding straws and other fibrous by-products for animals are as follows: (1) low protein content, (2) high crude fiber, (3) poor palatability and (4) low digestibility. Khilberg [1972] reported that even though these residues contain enough cellulose to make them an excellent source of energy for ruminants, they are poor quality feed in their natural state because of their low protein content. So researchers are trying to convert crop residues into useful chemical derivatives.

It is reported in previous that approximately 50 percent of the weight of the total corn plant is waste, consisting of stalks, leaves, cobs and husks that are already being utilized by many researchers given as below:

Rajagopal [1977] has reported on corn cob waste as a source of carbohydrate for the growth of fungi for use as animal feed in USA.

Lathrop [2007] has carried out many studies on the usage of corn cob in producing corncob pipe and biomass fuel source.

Loannidou *et al.* [2009] produced energy, fuel, materials and chemicals from corn residues such as cobs and stalks by non-catalytic and catalytic pyrolysis in two reactors.

Burrouohs *et al.* [1945] evaluated the nutritive value of corn cobs as feed in cattle and other ruminants.

Mullen *et al.* [2010] produced bio-oil and bio-char from corn cobs and corn stover [stalks, leaves and husks] by fast pyrolysis using a pilot scale fluidized bed reactor.

Nidhee and Sharma [2005] produced citric acid and single cell protein [SCP] from enzymatic hydrolysates of corn stover containing fermentable sugars equivalent to 10% [w/v] glucose.

Liming and Xueliang [2004] have prepared high-yield cellulose by using *Trichoderma reesei* ZU-02 on corn cob residue.

Kuan *et al.* [2011] prepared dietary nanofibres from corncob and wheat straw, and also studied physicochemical characteristics of alkali treated.

Muthusamy *et al.* [2012] have developed a technique for removal of nickel ion from industrial waste water by using corn cob.

Caliphs *et al.* [2010] and Zvinowanda *et al.* [2009] have demonstrated the usefulness of corn tassel as an alternative adsorbent for the removal of heavy metals such as Pb[II], chromium [VI] and cadmium [II] ions from aqueous solution

Oseni and Ekperigin [2007] have produced energy rich animal feeds from agricultural waste products of corn cobs and shafts by microbial conversion using

Aspergillus niger for 72 hours and reduced the pollution effects of these wastes during corn seasons.

Eze and Ojike [2012] had undertaken a study on the anaerobic digestion of corn wastes. Results obtained showed that corn chaffs, stalks and cobs have the potential to generate biogas, but the chaffs produced more biogas than the other two wastes.

Ohwoavworhua *et al.* [2005] have prepared low crystallinity cellulose from corn-cob and determined some physicochemical properties.

Ochetim S. [1993] has determined the feeding and economic value of corn cob meal for broiler chickens.

Ketcha *et al.* [2012] have prepared and characterized of activated carbons utilizing corn cobs by zinc chloride activation.

Tsai *et al.* [2001] also has prepared activated carbons from corn cob catalyzed by potassium salts and subsequent gasification with CO_2.

Israel *et al.* [2008] have synthesized cellulosic polymers namely cellulose, di- and triacetate form stem and cob of corn plant [*Zea mays* L.].

Though some researchers have done some works on corn waste, but a literature search found no published reports for producing food grade cellulose derivatives from corn waste elsewhere in the country or abroad.

1.7 Cellulose

As corn wastes contain good amount of cellulose, it can easily be used as the raw material for producing food grade cellulose derivatives with low cost. Cellulose is a linear, high molecular weight biodegradable polymeric material produced by nature at an annual rate of $10^{11} – 10^{12}$ tons [Hon, 1994].

Cellulose is the major polymer in lignocellulosic biomass [35– 48%] [Blaschek and Ezeji, 2007], and exists of D-glucose subunits, linked by β-1,4 glycosidic bonds [Kumar *et al.,* 2009; Hays *et al.*, 2008]. Each unit is rotated through 180° with respect to its neighbors, so that the structure repeats itself every two units (**Figure 1.1**). Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises the major proportion of cellulose, whereas a small percentage of unorganized cellulose chains form amorphous cellulose. Cellulose is more susceptile to degradation in its amorphous region [Beguin and Aubert, 1994].

Cellulose fibres are embedded in a lignin-hemicellulose matrix and this property contributes to the recalcitrance of lignocellulosic biomass to hydrolysis. Therefore, pre-treatment of lignocellulosic biomass before hydrolysis is a vital step [Blaschek and Ezeji, 2007].

The relative reactivity of the hydroxyl groups of cellulose has been studied and result showed cellulose reacts as a trihydric alcohol with one primary and two secondary hydroxyl groups per glucose unit [Ali *et al.*, 1999].

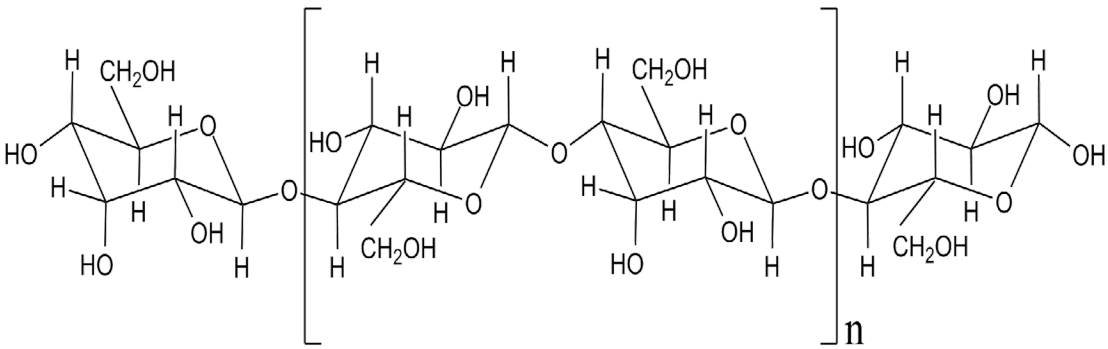


Figure 1.1 Cellulose molecule

1.8 Chemical modification of cellulose

Cellulose can be modified by chemical reactions (esterification, etherification and oxidation) in several ways to produce cellulose derivatives [Heinze, 1998]. Production of cellulose derivatives is done by reacting the free hydroxyl groups in the anhydroglucose units (AGU) with various chemical substitution groups. The introduction of the substituent disturb the inter- and intramolecular hydrogen bonds in cellulose, which leads to liberation of the hydrophilic character of the numerous hydroxyl groups and restriction of the chains to closely associate [Togrul and Arslan, 2003]. However, substitution with alkyl groups reduces the number of free hydroxyl groups. Unlike other natural polymers, cellulose derivatives have low water solubility due to formation of hydrogen bonding between hydroxyl groups in its side chains, leading to holding of the cellulose backbone chains together [Vlierberghe *et al.*, 2011; Ramanan *et al.*, 2006].

Cellulose is commonly converted into useful derivatives by etherification. Cellulose etherification is a very important branch of commercial cellulose derivatization, generating products that, in solution, increase the viscosity of the medium. Therefore, most of cellulose ethers are used as thickeners. In 1905, the first cellulose ether was synthesized: methellulose, widely used nowadays in food and cosmetic products and also as a treatment of constipation since it is not toxic and not allergenic. After 1920, the synthesis of carboxymethyl cellulose and hydroxyethyl cellulose were described [Balser *et al.*, 1986].

Carboxymethyl cellulose (CMC), cellulose nitrate, cellulose acetate are important cellulose derivatives which have been either fabricated into a hydrogel or

drawn into fibres for textile applications and composite materials for safety glass [Lee *et al.*, 2015].

Among these, Carboxymethyl cellulose is the most important and most widely used commercial water soluble derivative. Due to the importance of its multiple applications, CMC and its sodium salt are commercially produced in much higher amounts than any other cellulose ether [Mohkami and Taleipur, 2011].

1.9 Carboxymethyl cellulose

Carboxymethyl cellulose (CMC) is most important artificial-nature polymer derived from cellulose. This water-soluble polymer was invented in 1918 and was produced commercially in the early 1920's at the IG Farbenindustrie AG in Germany [Balser *et al.*, 1986], with the first patent being granted in 1921 (DE 332203). However, since then, significant improvements in process technology, in product quality, and in production efficiency have been made. A historical overview of CMC production on an industrial scale including comments about the future development of this important cellulose derivative was recently published [Stigsson *et al.*, 2001]. At present, the annual worldwide production of CMC is 300,000 tons [Jaderby *et al.*, 2005].

CMC is a copolymer of two units: ß–D–glucose and ß–D– glucopyranose 2–O–[carboxymethyl]–monosodium salt, not randomly distributed along the macromolecule, which are linked via ß–1,4–glycosidic bonds. The substitution of the hydroxyl groups by the carboxymethyl group is slightly preponderant at C–2 of the glucose [Charpentier *et al.*, 1997] (**Figure 1.2**). Hydroxyl groups in cellulose are

usually replaced by carboxymethyl groups in the order of C_6> C_2> C_3 [Ho and Klosiewicz, 1980; Reuben and Conner, 1983].

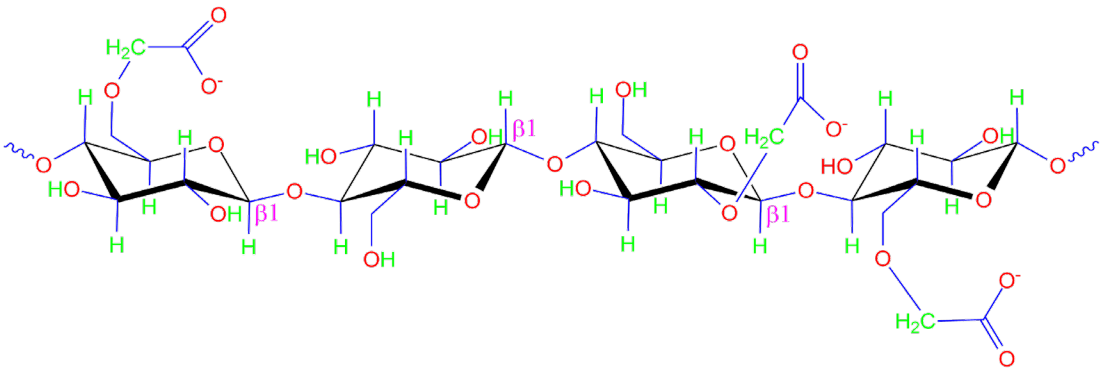
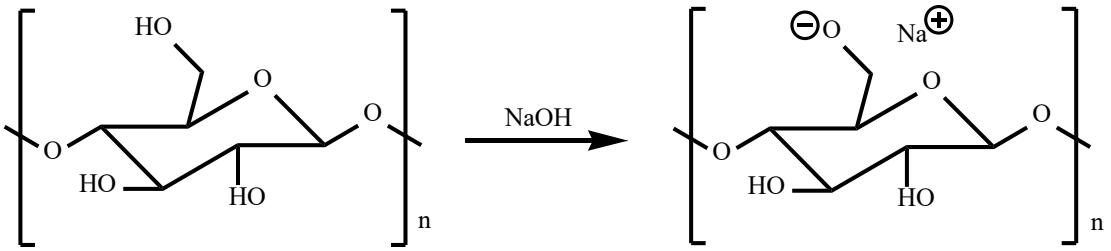


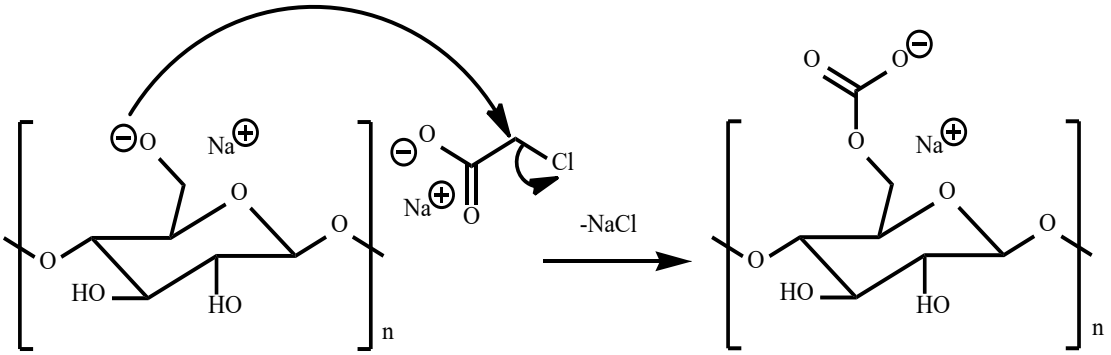
Figure 1.2 Structural unit of CMC

CMC is manufactured by reacting monochloroacetic acid with alkali cellulose. It is almost always distributed as the sodium salt, but instead of using the longer name, sodium carboxymethyl cellulose (NaCMC), it is usually designated simply carboxymethyl cellulose (CMC) [Stigsson *et al.*, 2006]. In this thesis, NaCMC or sodium carboxymethyl cellulose is used as a short name for CMC or carboxymethyl cellulose. CMC is produced by Williamson etherification in an aqueous-alcoholic system according to the following reactions [Stigsson *et al.,* 2001]:

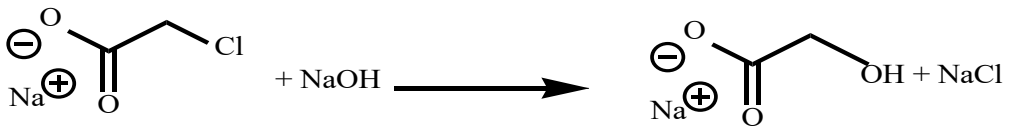
Mercerization



Etherification



Side reaction



The preparation of CMC involves two reaction stages: mercerization and etherification. One of the most important properties of the cellulose raw material used in the manufacture of CMC is its reactivity. The literature shows that approximately 35 to 50% of the hydroxyl groups can be etherified in cotton or dissolving pulps [Krassig, 1993]. Carboxymethylation is a uniform reaction it occurs only at accessible hydroxyl groups, followed by the slow penetration of the ordered surfaces [Borsa *et al.,* 1992].

Cellulose reactivity in a heterogeneous alkali medium depends not only on the cellulose structure at molecular, supermolecular and morphological levels, but also on the reaction medium employed. It is known that the reaction rate in ethanol is lower than that in isopropanol since ethanol is better at dissolving sodium hydroxide [Olaru and Olaru, 2001]. The crystallinity and polymorphism of the cellulose change during the mercerization step; the organic solvent acts as a swelling-restrictive agent and does not permit full hydration of the cellulose chain [Yokota, 1985].

Isopropanol is a poorer solvent for sodium hydroxide compared with ethanol, and a two-phase system therefore occurs. Only small amounts of the Na^+ and the OH^- ions enter the alcohol phase, favoring a higher concentration of NaOH in the vicinity of the cellulose. This results in substantial decrystallization and change of polymorphism from cellulose to Na- cellulose during mercerization. It is also reported that different solvent systems affect the characteristics of CMC during its manufacture. For example, when isopropanol is present during the mercerization stage substitution is more uneven; tri-substituted units occur and substitution on the C_6 carbon is increased [Stigsson *et al.,* 2006]. Industrially, the semi-dry ethanol process is normally used. However in the laboratory other organic solvent are appropriate and thus routinely employed [Heinze *et al.,* 1989].

Physical and chemical properties of CMC are mainly signified by the DS. The maximum DS is 3 [Salmi *et al.*, 1994]. To obtain higher DS through carboxymethylation, researchers have focused on certain factors, such as the solvent system, concentration of alkali, MCA, temperature, duration of reaction, and the steps of carboxymethylation [Khullar *et al.*, 2005]. However, a literature search found no published reports of producing a higher DS by single-step carboxymethylation than by multistep

carboxymethylation. So, study is necessary to produce a higher DS by single-step carboxymethylation, rather than multistep carboxymethylation.

1.10 Characteristics of CMC [Mario *et al.*, 2005; Keller, 1986; Hattori *et al.*, 2004]

1. White powder or micro-yellow fibrous in pure state.
2. Almost odourless and tasteless
3. Hygroscopic powder
4. Very fine particles
5. Finely distributed molecular weight
6. High resistance to acid
7. High resistance to salt
8. High transparency, low free fibres
9. Non -toxic
10. Free- flowing powder with fine granules

1.11 Applications of CMC

Carboxymethyl celluloses (CMC) are important industrial polymers with a wide range of applications in detergent, food, textile, pharmaceuticals, cosmetics and paint industries [Methacanon *et al.*, 2003]. There are several types of CMC grade depending on their applications such as technical, semi-purified and purified.

Large quantities are produced in crude commercial grades without any refining for use as a constituent in detergents, adhesives, pesticide, lubricants, cloth, cement, ceramics, oil drilling mud, and in the paper and coating industry [Doltz *et al.*, 2007]. Purified CMC is used in a variety of industries including the food, detergents, personal care, pharmaceutical etc [Stigsson *et al.*, 2001; Heydarzadeh *et al.*, 2009].

In textile industry CMC is applied as a dye thickening agent [Fijan *et al.*, 2009].

In detergent and surfactants it is used as anti-dirt agent for protection of fibres surface [Mohanty *et al.*, 2003]. CMC can improve detergency.

In pharmaceuticals, CMC have been employed as a biomaterial such as tissue engineering, drug delivery and other medical materials [Lee *et al.*, 2015]. It is also used in gels applied as protecting agents during heart, thorax and cornea surgery [Pennell *et al.* 1992]. CMC gives good enteric coatings for powders and tablets [Dapia *et al.*, 2003]. It is a key component in controlled drug-release pills and in the manufacture of personal care products [Maile, 1990].

In paper industry, CMC is often used for improvement of paper quality, as it can participate in the development of bonds between the cellulosic fibres. The film coated on the cellulose fibres by CMC gel has enhanced physical property for printing purposes [He *et al.*, 2009].

1.12 CMC in food industry

Especially the highly purified products, i.e., of low salt content have made the CMC to a valuable additive in many areas of application in the food area. CMC can thicken, stabilize and improve taste. CMC is widely used in frozen desserts, protein food, beverages, icings, dressings, bread, cake, biscuit, instant noodles etc. as various properties given below:

1. Thickening: CMC can produce high viscosity at low concentration. It also acts as lubricant.
2. Water retention: CMC is a water binder, helps increase shelf life of food.
3. Suspending aid: CMC acts as emulsifier and suspension stabilizer, particularly in icings to control ice crystal size.

1. Film forming: CMC can produce a film on the surface of fried food, e.g. instant noodle, and prevent absorption of excessive vegetable oil.
2. Chemical stability: CMC is resistant to heat, light, mold and commonly used chemicals.
3. Physiologically inert: CMC as a food additive has no caloric value and cannot be metabolized.

1.13 Agro cellulosic source of CMC

The global agriculture sector produces billions of tons of agricultural biomass, such as cereals, oil seeds and other plant commodities, annually. However, this progress has also led to the generation and accumulation of agricultural wastes with little commercial value. Only a small fraction of agricultural wastes are used, as animal feeds and for energy production [Sain and Panthapulakkal, 2006].

There are many researches which studied the production of CMC from agricultural waste as a cellulose sources. CMC from sugar beet pulp cellulose and rheological behaviour of CMC was studied by Togrul and Arslan [2003]. Adinugraha *et al.* [2005] synthesized and characterized sodium CMC from cavendish banana pseudo stem (Musa cavendishii LAMBERT). They found, in alkalization, using 15% NaOH provided the highest DS value of CMC.

Pushpamalar *et al.* [2006] investigated the optimization of reaction conditions for preparing CMC from sago waste. They presented the highest DS value was obtained from using isopropyl alcohol as a solvent and 25% of NaOH concentration in alkalization.

Rachtanapun *et al.* [2007a] studied the production of CMC films from papaya peel. Also, Rachtanapun *et al.* [2007b] studied the effect of blend CMC from papaya

peel / corn flour films on mechanical properties and WVTR. Rachtanapun *et al.* [2007c] studied the synthesis of CMC and CMC films making from waste of mulberry paper. In addition, Rachtanapun *et al.* [2007d] also studied the effect of bleaching process on mechanical properties of CMC from papaya peel. They found the percentage of hydrogen peroxide increased with increasing of tensile strength, elongation at break but folding endurance decreased.

Waring and Parsons [2001] reported the DS of CMC is depended on the monochloroacetic acid concentration, reaction temperature and duration of reaction. The solubility of CMC increases with increasing of DS. Moreover, Siralertmukul *et al.* [2005] studied the modification of CMC from durian husk and the carboxymethylation was carried out under different of monochloroacetic acid which indicated the DS value and the highest DS value of CMC as 0.67.

There are also some other agricultural wastes, such as, rice straw, sugarcane bagasse, saw dust, cotton staples [Hebeish *et al.,* 1984], orange mesocarp [Akaranta and Osugi, 1997], and weeds, *Eichoria crassipes* [Barai *et al*., 1997] have been used as a base material for production of CMC differing in their DS and properties using different set of reaction conditions depending upon the DP and composition of the cellulosic material.

But there were no information obtained for the preparation of CMC and food-grade CMC from corn waste through the studies of literature.

Nowadays, the attention of many scientists is focused on the development of new methods for synthesis and stabilization of food-based products from agro cellulosic sources. Preparation of food- grade CMC from different parts of corn waste

can be considered as a feasible alternative way for generating such value-added products.

1.14 Safety assessments of CMC by regulatory agencies

A comprehensive search of the scientific and regulatory literature was conducted to assess safety-in-use of carboxymethyl cellulose. The safety of cellulose and cellulose derivatives, including carboxymethyl cellulose as food additives has been extensively evaluated by regulatory bodies including the US Food and Drug Administration (FDA) [21 CFR 182.90- Substances migrating to food from paper and paperboard products], the WHO/FAO Joint Expert Committee on Food Additives (JECFA), the former European Union Scientific Committee on Food (SCF), and the European Food Safety Authority (EFSA).

In accordance with 21 CFR § 170.30, the intended use of carboxymethyl cellulose has been determined to be generally recognized as safe (GRAS) based on scientific procedures. Several cellulose derivatives, including carboxymethyl cellulose have been permitted for food uses as codified in 21CFR. JECFA has assigned a group acceptable daily intake (ADI) as “not specified” for seven modified celluloses, including carboxymethyl cellulose. This indicates that carboxymethyl cellulose as part of a group ADI established for these celluloses when used as food additives will not have adverse effects on human health at any point in a person’s life, even if they are consumed daily.

In addition to these regulatory assessments, there are several scientific studies on cellulose derivatives, including carboxymethyl cellulose. There is sufficient qualitative and quantitative scientific evidence, including human and animal data, to determine safety-in-use for carboxymethyl cellulose. The safety determination is

based on the totality of available evidence, including animal, human, and in vitro studies conducted with carboxymethyl cellulose as well as other cellulose derivatives. The totality of the available evidence suggests that the estimated daily intake of carboxymethyl cellulose 30 g/person/day from the proposed uses, if ingested daily over a lifetime, is safe [FDA, 2013].

The following cellulose derivatives are listed either as GRAS or permitted for direct addition to food as food additives:

1. Carboxymethyl cellulose;
2. Methyl cellulose;
3. Cellulose acetate;
4. Ethyl cellulose and
5. Hydroxypropylmethyl cellulose.

Carboxymethyl cellulose (water absorbing characteristics) is approved as a multipurpose additive for its use as a binder and filler, as a whipping agent, in ice-cream, confectionery, jellies etc [FDA, 2013].

Toxicological and biological role of several cellulose derivatives, including carboxymethyl cellulose were investigated by researchers and reviewed by national and international regulatory agencies such as FDA, EFSA, JECFA etc. In general, no adverse toxicological effects for cellulose ethers have been reported [FDA, 2013].

When a chemical substance is administered to a biological system, different types of interactions can occur and a series of dose -related responses may result. In most cases these responses are desired and useful, but there are a number of other effects which are not advantageous. Therefore, a comprehensive search of the scientific literature was needed to conduct relevant safety studies of the prepared CMC.

Today, food and colour additives are more strictly regulated than at any time in history. So, extensive analysis for potential external contaminants of CMC such as heavy metals and microbial contaminants is necessary, so that the product complies with the purity requirements of Joint FAO-WHO Expert Committee on Food Additives [JECFA, 2002].

In recent times there is an increasing awareness and interest in agro-based waste products for food industry. The major hindrance to the use of agrochemicals is the lack of scientific and clinical data in support of better understanding of the efficacy and safety for human health. So, detail study was needed to assess the safety of synthesized CMC for use as a food ingredient or dietary supplement at the levels prescribed by national and international regulatory and other agencies.

1.15 Thesis objectives

Every year in our country, approximately 3000-4000 tons CMC is being imported in lieu of valuable foreign currencies. But still now we have no local industry in producing CMC of any grade. Corn husk is one of the largest biomass generated from corn-processing areas, and is often left unutilized in harvested fields. These materials are accumulated in enormous amounts in Bangladesh alone and serve as source of environmental pollution.

No project work on corn husk for preparing food-grade CMC or in the related field has yet been implemented elsewhere in the country. But this agro waste can easily be collected with free of cost and can be used to produce food-grade CMC.

Today's in the world, more and more attention has been paid to prepare environmentally friendly agro-based food products due to rapid increasing of industrialization and population as well as due to the increasing environmental concerns.

In the view of these perspectives, the following parameters were aimed in my research work:

- The first aim of this research was to treat cellulosic and lignocellulosic materials to get high quality α-cellulose. This aim has been achieved through the investigation of corn waste such as husks, cobs, leaves, stalks etc.

- The second objective of this research was to optimize the reaction condition in terms of cellulose particle size.

- The third aim of this research was to prepare highly substituted CMC which was achieved by applying size reduction process of cellulose.
- The fourth main broad objective of this research was to synthesis food-grade CMC from corn husk cellulose.
- The fifth objective of the study was to evaluate the systematic toxic effect of the prepared CMC in Swiss albino mice and also to determine the No Observed Adverse Effect Level [NOAEL].

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CHAPTER 2

PREPARATION AND CHARACTERIZATION OF CMC

2.1 INTRODUCTION

Continuously depleting limited fossil supplies, rising price, problem of non-biodegradability of petroleum-based polymers, and the recent environment-conservative regulations have triggered search for non-conventional sources of cellulose biomass as feedstock for production of cellulose and its derivatives. Having initiated efforts in this direction, isolation and characterization of cellulose and its derivatives from non-conventional biomass were studied. The study contributed to find appropriate conditions for production of α-cellulose from corn waste and its carboxymethylated derivative such as CMC from corn husk cellulose.

This chapter reports a method of converting the unused corn husk to commercially-useful and high-quality food-grade CMC. The composition of corn waste such as cobs, husks, leaves, stalks etc investigated for CMC production are also described in this chapter. The effect of solvent system, particle size, alkali concentration for basification, concentration of monochloroacetic acid for etherification, time and temperature of the reaction for the degree of substitution are studied.

The synthesis of CMC from corn husk waste was carried out according to the **Scheme-1**. The synthesized product analyzed for purity, DS, yield, molar mass and solubility are given in the chapter. Microbiology of our prepared CMC is also described elaborately in this chapter. The novelty of present work was to achieve higher degree of substitution at single step carboxymethylation instead of multisteps carboxymethylation by applying particle size technique, and also the potentiality of the process was the re-use of ethanol after washing of crude CMC.

2.2 MATERIALS AND METHODS

2.2.1 Materials

All the chemicals used in the present investigation are listed below with their sources and purity.

Chemicals	**Producer**
Ethyl alcohol (C_2H_5OH, 95%)	Merck (Germany)
n-hexane (C_6H_{14}, 95%)	Merck (Germany)
Ammonium carbonate $(NH_4)_2CO_3$, 99.5%	Merck (Germany)
Ammonium oxalate $(NH4)_2C_2O_4$, 99%	Merck (Germany)
Sulfuric acid (H_2SO_4, 98%)	Merck (Germany)
Sodium chlorite($NaClO_2$, 80%)	Merck (Germany)
Potassium dichromate ($K_2Cr_2O_7$, 99%)	Merck (Germany)
Sodium hydroxide (NaOH, 98%)	BDH (England)
Monochloroacetic acid ($ClCH_2COOH$, 98%)	BDH (England)
Sodium carbonate (Na_2CO_3, 99.5%)	Merck (Germany)
Standard carboxymethyl cellulose (DS 0.8, 99%)	BDH (England)
Sodium silicate ($Na_2O_3Si.\ 9H_2O$, 98%)	Merck (Germany)
Di-phosphorous pentoxide (P_2O_5, 97%)	Merck (Germany)
Hydrochloric acid (HCl, 36.5%)	BDH (England)
Methanol (CH_3OH, 99.8%)	Merck (Germany)
Nitric acid (HNO_3, 65%)	Merck (Germany)
Silver nitrate ($AgNO_3$, 99%)	BDH (England)
Potassium bromide (KBr, 99%)	Merck (Germany)
Sodium meta bisulfite anhydrous ($Na_2S_2O_5$, 99%)	Merck (Germany)

Chemicals	**Producer**
Acetone (CH_3COCH_3, 99%)	Merck (Germany)
Glacial acetic acid (CH_3COOH, 99.99%)	Merck (Germany)
2,7-dihydroxynaphthalene ($C_{10}H_6(OH)_2$, 98%)	Merck (Germany)
Glycolic acid ($HOCH_2COOH$, 99.5%)	Merck (Germany)
Toluene (C_6H_5-CH_3, 99%)	Merck (Germany)
Dextrose ($C_6H_{12}O_6$, 99 %)	Merck (India)
Sodium chloride (NaCl, 99.5%)	Merck (Germany)
Lactose monohydrate ($C_{12}H_{24}O_{12}$, 99%)	Merck (Germany)
Agar powder $(C_{12}H_{18}O_9)_n$, GPR	Sigma (Switzerland)
Mannitol ($C_6H_{14}O_6$,95%)	Merck (Germany)
Neutral red ($C_{15}H_{17}N_4Cl$, 90%)	Sigma (Switzerland)
Crystal Violet ($C_{25}H_{30}N_3Cl$, 90%)	Sigma (Switzerland)
Peptone (Bacteriological grade)	HiMedia (India)
Yeast extract (Bacteriological grade)	HiMedia (India)
Tryptone (Bacteriological grade)	HiMedia (India)
Sodium citrate ($C_6H_5Na_3O_7$, 99%)	Merck (Germany)
Sodium deoxycholate ($C_{24}H_{39}NaO_4$, 98%)	Merck (Germany)
Potassium phosphate (dibasic) (K_2HPO_4, 98%)	Merck (Germany)
Potassium phosphate (monobasic) (KH_2PO_4, 99%)	Merck (Germany)

2.2.2 Methods

- **Collection of cellulosic wastes**

Corn waste, a cellulosic waste of corn plants; such as leaves, husks, cobs and stalks was collected from Bangladesh Regional Wheat Research Institute (BRWRI) Regional Station, Rajshahi with free of cost.

- **Preparation of sample**

At first locally collected corn husk sample was sorted manually to remove defective parts and foreign materials, washed with distilled water. The fresh and clean corn husks were taken, cut manually into small pieces and dried in the sun to remove moisture. The dried sample was ground into powder using a grinding disk mill (model: FFC-15, China). The powdered sample was then sieved (Sieve type: OHIO 44060, USA) and separated into different particle sizes by passing the sample through sieves of different mesh sizes such as 10, 44, 100, 150 and 200; and stored in a desiccator for compositional and chemical analysis.

- **Isolation and estimation of constituents of sample**

The compositional data of waste samples such as α-cellulose, hemicellulose (β and γ-celluloses), Klason lignin, pectic matters, and fatty and waxy matters, etc (as the average of four replicate analyses) was determined. The results were expressed as weight percent and calculated according to a TAPPI standard [1993], and Mondal and Haque's method [2007]. The other fractions including uronic acids, soluble lignin, acetyl groups etc were not determined owing to their minor importance for the purpose of this work.

Estimation of aqueous extracts

The powdered samples were treated with distilled water at 100°C for 2 h. Then they were filtered and dried at 105ºC until reaching a constant weight. The loss in weight gave the amount of aqueous extracts as,

$$\text{Aqueous extracts, \%} = \frac{W_1 \times 100}{W_0}$$

Where, W_1 and W_0 are weight loss and the initial weight respectively of the sample.

Estimation of fatty and waxy matters

The sample was immersed in a n-haxane-alcohol mixture (2:1 v/v), in the solid to liquid rkatio of 1:100. Then the sample was allowed to stand for 10 h with occasional stirring.

The sample was washed with fresh n-hexane: alcohol mixture and finally with alcohol. The sample was then dried at 105°C until reaching a constant weight. The loss in weight gave the amount of fatty and waxy matter estimated according to this equation,

$$\text{Fatty and waxy matters, \%} = \frac{W_1 \times 100}{W_0}$$

Where, W_1 and W_0 are weight loss and the initial weight respectively of the sample.

Determination of pectic matters

The defatted and dewaxed sample was heated with 0.5% ammonium oxalate solution in the solid-to-liquid ratio of 1:100 at 70-80°C for 3 days on a heating mantle. As evaporation continued, hot distilled water was added to keep the level of solution constant throughout the process. The sample was filtered, washed thoroughly

with hot distilled water and then dried at 105°C until maintaining a constant weight. The loss in weight was used to estimate the amount of pectic matters according to the following formula,

$$\text{Pectic matters, \%} = \frac{W_1 \times 100}{W_0}$$

Where, W_1 and W_0 are weight loss and the initial weight respectively of the sample.

Determination of Klason lignin

The dewaxed and depectinized sample was dried at 105°C, and treated in 72% sulfuric acid in a solid-liquid proportion of 1:15. The solution was stirred frequently at ordinary temperature. The mixture was allowed to stand for 2 h, and then diluted to 3% acid concentration. After refluxing for 4 h, the mixture was allowed to stand overnight. Then it was filtered through a sintered glass funnel and washed thoroughly with hot distilled water. The constant weight of the residue in the sintered funnel, dried at 105°C, was estimated as the amount of the lignin content**.**

Estimation of α-cellulose and hemicellulose

In this step, all non-cellulosic matters were removed from the sample by a treatment with bleaching agents such as $NaClO_2$. Chlorite holocellulose, a combination of α-cellulose and hemicelluloses was obtained, and was taken as the total in the sample.

Preparation of chlorite holocellulose

A suitable amount of dewaxed and depectinized sample was dried at 105°C and treated with a 0.7% $NaClO_2$ solution. This solution was then buffered to pH 4, in a solid-to-liquid ratio of 1:50, at 90-95°C for 90 min. After filtering and washing the

sample, it was then treated with 0.2% sodium meta-bisulphite solution for 15 min. Then the sample was again filtered and washed thoroughly with distilled water. Finally, the sample was dried at 105°C until reaching a constant weight.

Determination of α- cellulose and hemicellulose

The dried chlorite holocellulose sample was placed in a 250 ml beaker and 75 ml 17.5% NaOH was added at room temperature. The time was noted at which the reagent was added and allowed to stand a few minutes until the sample was absorbed. Then the sample was stirred with a glass rod until it was dispersed. When the sample was dispersed, the stirrer was rinsed with another 25 ml 17.5% NaOH reagent, adding it to the beaker, so that exactly 100 ml of the reagent had been to the sample. After a period of 30 min from the first addition of the NaOH reagent, 100 ml of distilled water was added to the sample suspension and stirred thoroughly with a rod.

At the end of the 60 min period, the sample was transferred to a filtering funnel, then collected about 150 ml of the filtrate in a clean and dry filtration flask. 20 ml of the filtrate and 10 ml of 0.5 N potassium dichromate solutions were pipetted into a 250 ml flask. Cautiously 50 ml of concentrated H_2SO_4 was added into the flask. The solkution was allowed to remain hot for 15 min, and then 50 ml of water was added and cooled to room temperature. 2 drops of ferroin indicator was added and titrated with 0.1 N ferrous ammonium sulphate solutions to a purple colour. A blank titration was made substituting the sample filtrate with 10 ml of 17.5% NaOH and 10 ml of water.

Determination of β- and γ-celluloses

50 ml of the filtrate sample was pipetted into a 100 ml graduated cylinder having a ground glass stopper. 50 ml of 3N H_2SO_4 was added and mixed thoroughly by inverting. The cylinder was heated in hot water bath at about 70-90ºC for a few minutes to coagulate the β- cellulose. The precipitate was allowed to settle for overnight, then filtered to obtain a clear solution. 40 ml of the clear solution and 10 ml of 0.5N $K_2Cr_2O_7$ were pipetted into a 250 ml flask and cautiously 90 ml of concentrated H_2SO_4 was added into the flask. The solution was allowed to remain hot for 15 min, proceed with titration as outline before. A blank titration was made substituting the solution with 10 ml of 17.5% NaOH, 10 ml of water and 20 ml of 3N H_2SO_4.

Calculations

α –, β – and γ – celluloses were calculated by the following formula:

Hemicellulose (β – and γ – celluloses), % = $\frac{6.85\ (V_2\text{-}V_1) \times N \times 20}{A \times W}$ and

α – cellulose, % = 100 – $\frac{6.85\ (V_2\text{-}V_1) \times N \times 20}{A \times W}$

Where,

V_1 = Volume of the titrant for sample solution, ml (Ferrous ammonium sulphate solution)

V_2 = Volume of the titrant for blank solution, ml (Ferrous ammonium sulphate solution)

N = Exact normality of the ferrous ammonium sulphate solution

A = Volume of the filtrate sample used in the oxidation

W = Oven dry weight of sample, g

$$\gamma - \text{cellulose, \%} = \frac{6.85(V_4 - V_3) \times N \times 20}{A \times W}$$

Where,

V_3 = Volume of the titrant for solution after precipitation of β – cellulose, ml

V_4 = Volume of the titrant for blank solution, ml

β – cellulose, % = Hemicellulose % — (γ – cellulose %)

➢ **Preparation of carboxymethyl cellulose**

The main step of carboxymethylation is the formation of alkali cellulose which modifies the crystalline structure of cellulose. This form increases the accessibility of the sample to chemicals by swelling [Barba *et al.,* 2002].

The carboxymethyl cellulose was synthesized by the conversion of maize husk cellulose to alkali cellulose. This alkali cellulose was swollen in aqueous NaOH, and a surplus of 95% ethanol as solvent with monochloroacetic acid. The production of CMC was carried out by the following two steps:

- Basification

5 g of cellulose powder and 30% aqueous ethanolic NaOH (w/v) were mixed in the cellulose-liquid proportion of 1:2.7. The temperature of the mixture was carefully controlled at 30°C, for 1 h with occasional stirring. This step is known as "steeping".

$$[C_6H_7O_2(OH)_3]_n + nNaOH \rightarrow [C_6H_7O_2(OH)_2ONa]_n + nH_2O$$

- Etherification

In the second step, 120% (w/v) aqueous mono-chloroacetic acid ($ClCH_2COOH$) was added drop-by-drop to the mixture derived in basification step. A cellulose-liquid proportion of 1:1.2 was maintained and its temperature was carefully controlled at 55°C for 3.5 h. This step is known as the "carboxymethylation" and the reactions are as follows:

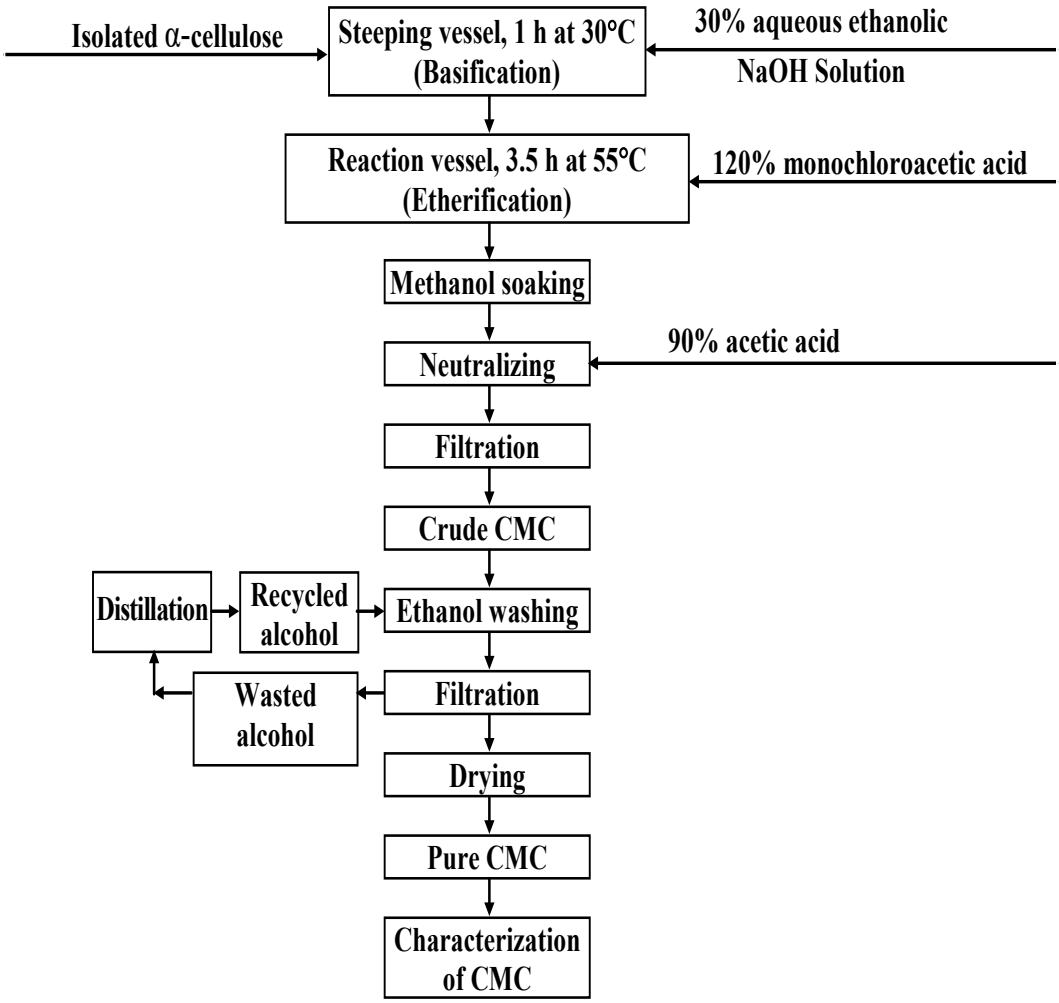
$$nClCH_2COOH + nNaOH \rightarrow nClCH_2COONa + nH_2O$$

$$[C_6H_7O_2(OH)_2ONa]_n + nClCH_2COONa \rightarrow [C_6H_7O_2(OH)_2OCH_2COONa]_n + nNaCl$$

There is also a side reaction as:

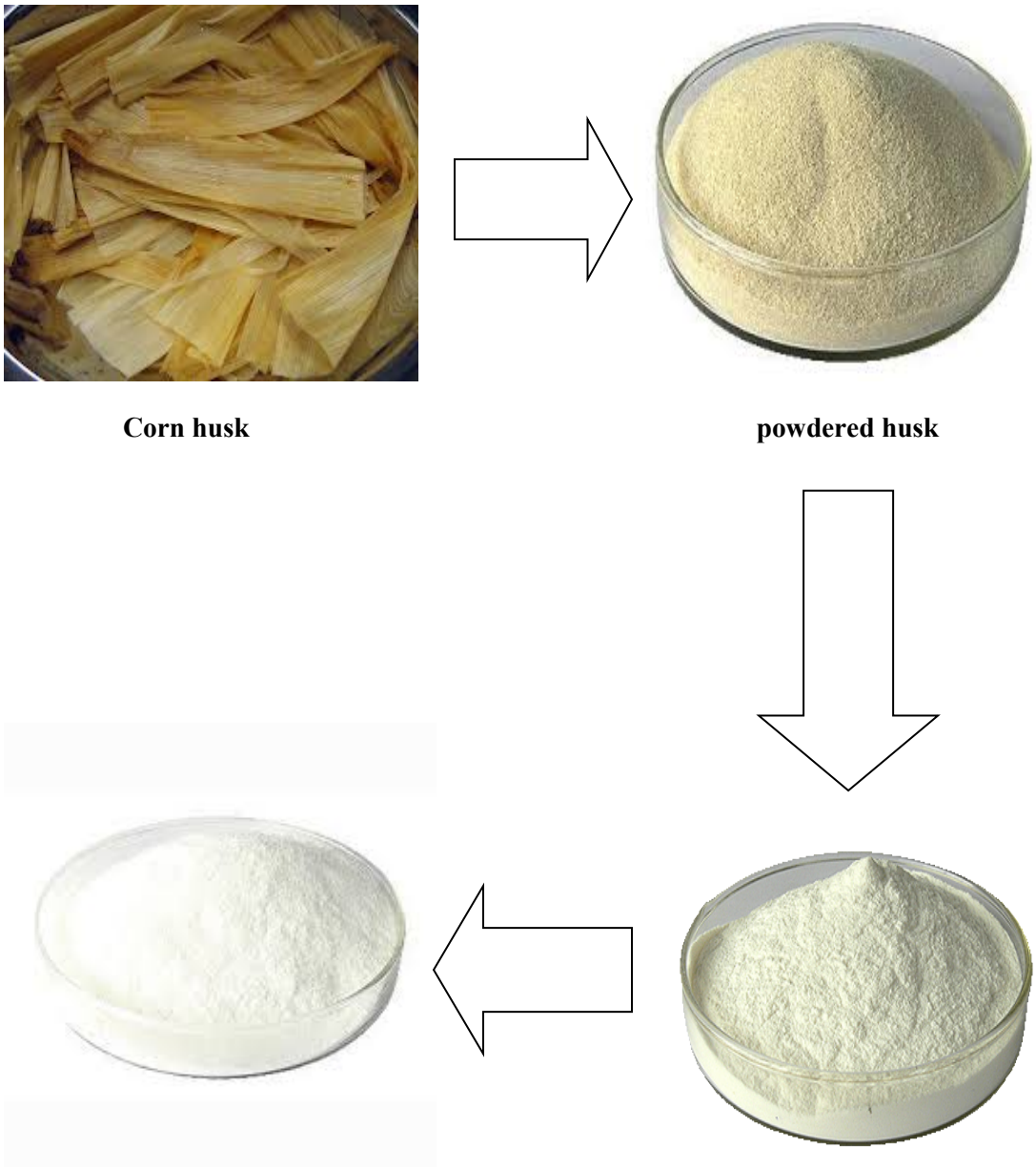
$$NaOH + ClCH_2COONa \rightarrow HOCH_2COONa + NaCl$$

After the completion of these reactions, 70% methanol (v/v) was added to the reactants and the mixture was neutralized with 90% acetic acid (v/v). CMC was then recovered by filtration and washed six times with ethanol/water (70/30 v/v). Finally, the product was oven dried at 60°C.



Scheme 1. A flowchart summarizing the key steps in producing CMC from α-cellulose of corn husk

Flow diagram of CMC preparation



- **Degree of substitution**

To determine the degree of substitution (DS), 0.5 g of dried CMC was ashed gently between 450 and 550°C for 24 h, and then dissolved in 100 ml of distilled water. 20 ml of this solution was titrated with 0.1 N sulphuric acid using methyl red as an indicator. After the first end point, the solution was boiled and titrated to a sharp end point. The degree of substitution of the carboxymethyl content [Abel *et al.,* 2001] was calculated as follows:

$$\text{Degree of Substitution (DS)} = \frac{0.162\times B}{1-0.08\times B}$$

Where, $B = \frac{0.1\times b}{G}$, b is the volume (in ml) of 0.1 N sulphuric acid and G is the mass of pure CMC in grams.

- **CMC yield**

CMC yield was measured on a dry weight basis. The net weight of dried CMC was divided by the weight of dried cellulose to get the yield value [Rachtanapun, 2009] as follows:

$$\text{CMC yield, \%} = \frac{\text{Weight of obtained CMC}}{\text{Weight of dried cellulose}}\times 100$$

- **Measurement of intrinsic viscosity of CMC**

In order to evaluate the molecular weight of polymeric chains, the intrinsic viscosity of the polymer is measured by using Ostwald viscometer in which viscosity of liquid is measured by comparing the flow times of two liquids of equal volumes using same viscometer.

CMC of four different concentrations (0.2, 0.4, 0.6 and 0.8%), was dissolved in 0.8 M NaOH solution. Ostwald viscometer was used to measure the passing time of the solutions through the capillary immersed in a warm water bath set at temperature of 25°C ±0.1°C. Three measurements were made on each sample. The running times of the solution and solvent were recorded as seconds and used to calculate intrinsic viscosity [Pamies *et al.,* 2008].

$$\eta_{rel} \text{ (Relative viscosity)} = \frac{t_2 \text{ (efflux time of solution)}}{t_1 \text{ (efflux time of solvent)}}$$

$$\eta_{sp} \text{ (Specific viscosity)} = \eta_{rel} - 1$$

$$\eta_{inh} \text{ (Inherent viscosity)} = \frac{\ln \eta_{rel}}{C}$$

$$\eta_{red} \text{ (Reduced viscosity)} = \frac{\eta_{sp}}{C}$$

$$\text{Intrinsic viscosity, } [\eta] = \lim_{C \to 0} \left(\frac{\eta_{sp}}{C}\right) = \lim_{C \to 0} \left(\frac{\ln \eta_{rel}}{C}\right)$$

Where, C = concentration of CMC solution (g/ml)

A plot was made of reduced viscosity on the *y*-axis, and concentration on the *x*-axis. The intrinsic viscosity (ml/g) was obtained by extrapolating reduced viscosity vs. concentration data to zero concentration.

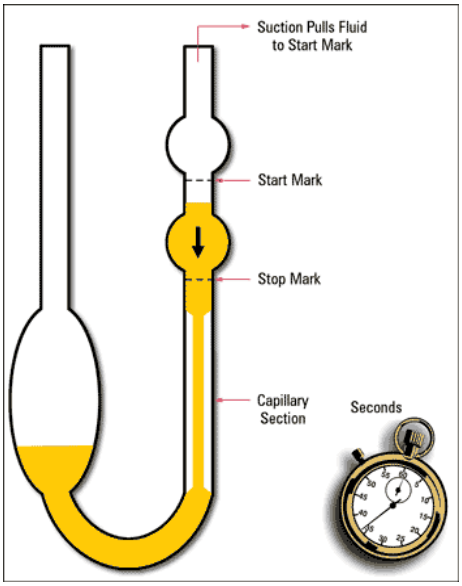


Figure: Ostwald viscometer

- **Molecular weight determination**

From the result of intrinsic viscosity, the molecular weight of the CMC was calculated by the "Mark-Houwink-Sakurada" equation [Brandrup *et al.,* 1998]. The Mark–Houwink equation gives a relation between intrinsic viscosity and molecular weight as,

$[\eta] = K M^{a}$

Where,

K = Constant for solvent;

a = Polymer shape factor;

[η] = Intrinsic viscosity;

M = Molecular weight of CMC.

- **Preparation of film with carboxymethyl cellulose**

The 3.0 g of CMC was dissolved in 100 ml of distilled water and heated at 80°C with constant stirring for 10 min to prepare the film forming solution. The solution was cooled down to 25°C and cast onto a cellophane plate (30 cm × 15 cm). Thickness of film was controlled by the volume of solution (70 ml) on each plate. This plate was left and dried at room temperature for 36 h. Then CMC film was obtained. The film was peeled off and kept in desiccator containing silica gel in the bottom to control the moisture content of film. The film was cut for property testing [Rachtanapun *et al.,* 2007].

- **Water holding capacity of the prepared CMC film**

The prepared 2% CMC film (6 cm × 4 cm) was weighed accurately and immersed in distilled water for 15 h at room temperature. The film was wiped using tissue paper and weighted again. Water holding capacity (WHC) was determined using the equation [Lin *et al.,* 2005] below as,

$$\text{Water holding capacity (WHC), \%} = \frac{(W_1 - W_0) \times 100}{W_0}$$

Where, W_0 (g) is the weight before immersion and W_1 (g) is the weight after immersion.

- **Oil holding capacity of the prepared CMC film**

The prepared 2% CMC film (6 cm × 4 cm) was weighed accurately and immersed in mustered oil for 15 h at room temperature. The film was wiped using tissue paper and weighted again. Oil holding capacity (OHC) was determined using the equation [Chau *et al.,* 1997] below as,

$$\text{Oil holding capacity (OHC), \%} = \frac{(W_1 - W_0) \times 100}{W_0}$$

Where, W_0 (g) is the weight before immersion and W_1 (g) is the weight after immersion.

- **Moisture content**

Porcelain crucible was first cleaned, heated at 105ºC, cooled and weighed. The sample was weighed and taken in a pre-weighted porcelain crucible. The crucible containing the sample was heated in an electrical oven (FC-610, Toyo Seisakusho Co. Ltd.) for about 6 h at 105ºC. It was then cooled in a desiccator and weighed. The process was repeated until weight was reached to constant [ICOMR, 1971].

$$\text{Moisture content, \%} = \frac{W_1 \times 100}{W_0}$$

Where, W_0 (g) is the weight of sample and W_1 (g) is the amount of moisture obtained.

- **Ash content**

Ash content was determined using standard methods [ASTM, 2002]. A crucible was weighed and ignited for 30 min at 600°C. It was then cooled and transferred into desiccator with the aid of tongs for 15-20 min and reweighed accurately. Approximately 0.5g of the vacuum dried sample was transferred into the crucible and the sample was pre-ashed in a fume hood. When the sample ceased giving off smoke, it was placed in a preheated 600°C muffle furnace for 6 h. When ashing was complete, the crucible was transferred directly into a desiccator, cooled and weighed. After completing the sample ashing, ash content was calculated using the equation below as,

$$\text{Ash content, \%} = \frac{W_1 \times 100}{W_0}$$

Where, W_0 (g) is the weight of sample and W_1 (g) is the weight of ash.

- **Gel content**

Gel content of the CMC films were determined by Soxhlet extraction using hot toluene as solvent. Films (6 cm × 4 cm) were weighed and placed in a cellulose extraction thimble in the Soxhlet extractor. The solvent extraction was carried out with 250 ml toluene for 6 h. After that, the sample was vacuum dried and re-weighed until it attained a constant weight. Gel content was calculated according to the following equation [Lin *et al.,* 2005] as,

$$\text{Gel Content, \%} = \frac{W_1 \times 100}{W_0}$$

Where, W_0 (g) is the weight before extraction and W_1 (g) is the weight after extraction.

- **CMC content**

Exactly 1.5 g of carboxymethyl cellulose (CMC) was added to 100 ml of 80% aqueous methanol solution, stirred, kept for 10 min and filtered. The cake was washed by the fresh 100 ml of 80% aqueous methanol and dried to obtain pure CMC [Togrul and Arslan, 2003]. The CMC content was calculated as,

$$\text{CMC content, \%} = \frac{100 \times M_2}{M_1}$$

Where, M_1 (g) is the weight of sample before wash and M_2 (g) is the weight of washed sample.

- **Sodium chloride content**

2 g of CMC was added to 250 ml of 65% aqueous methanol and kept for 5 h. 100 ml of liquid phase was neutralized by dilute 0.1N HNO_3 and titrated with 0.1N $AgNO_3$ solution [Togrul and Arslan, 2003]. The sodium chloride (NaCl) content was calculated as,

$$\text{NaCl content, \%} = \frac{1.461 \times V}{M}$$

Where, V (ml) is the amount of $AgNO_3$ solution and M (g) is the weight of dried sample.

- **Sodium glycolate content**

0.5 g of the sample was weight and transferred to a 100 ml beaker. The sample was moistened thoroughly with 5 ml of glacial acetic acid, 5 ml of water and stirred for about 15 min to ensure total hydration. Then 50 ml acetone and 1 g sodium chloride were slowly added to it and stirring was continued several minutes to ensure complete precipitation of CMC. It was then filtered through a fast filter paper previously washed with acetone into a 100 ml volumetric flask. The beaker was rinsed with 30 ml of acetone, filtered and made up to volume with acetone. The solution was allowed to stand for 24 h without shaking and clear supernatant was used to prepare the test solution.

A blank solution containing 5 ml of glacial acetic acid, 5 ml water and acetone in 100 ml volumetric flask was prepared. 2 ml each of the test and blank solutions were pipetted into separate 25 ml volumetric flasks. The acetone was removed by heating the uncovered flasks upright in a boiling water bath for exactly 20 min. Solutions were then cooled to room temperature, 5 ml of 2, 7-dihydroxynaphthalene

was added to each solution and mixed. Again 15 ml more of the 2,7-dihydroxynaphthalene was added to each solution and mixed. The mouth of the flask was covered with a small piece of aluminium foil and heated on a water bath for 20 min. Absorbance of the test solution against blank solution was measured at 540 nm wavelength using UV-visible spectrophotometer (model: T 60, England).

To make a calibration curve, 0.100 g of standard glycolic acid was weight accurately (previously dried in vacuum over phosphorus pentaoxide). It was then dissolved with water in a 100 ml volumetric flask and made up to volume with water. 0.0, 1.0, 2.0, 3.0, and 4.0 ml aliquots of standard glycolic acid solution were taken into a series of 100 ml volumetric flask. 5 ml water, 5 ml of glycolic acid were added to each flask, diluted to volume with acetone, mixed properly. Finally, 2 ml of each solution was taken into a series of 25 ml volumetric flasks. Then mg of glycolic acid in the original 100 ml solution against absorbance was plotted to give a calibration curve. This calibration curve of pure standard glycolic acid was used to determine the unknown concentration of sodium glycolate in a solution using following formula [JECFA, 2002] as,

$$\text{Sodium glycolate content, \%} = \frac{a \times 1.29}{b}$$

Where, 1.29 = factor of converting glycolic acid into sodium glycolate

a = mg of glycolic acid read from the calibration curve

b = dry weight of sample in g.

- **Swelling behaviour in water**

In a beaker with 30 ml distilled water at 30°C, 1 g of purified CMC was added and kept for several hours. Then the behavior of the CMC in the water was observed.

- **Atomic Absorption Spectroscopy analysis**

Atomic Absorption Spectroscopy (AAS) is a technique used mostly for determining the concentration of a particular metal element in a sample. AAS can be used to analyze the concentration of over 62 different metals in a solution.

The amount of arsenic (As), lead (Pb), cadmium (Cd) and mercury (Hg) in the prepared CMC were measured by Atomic Absorption Spectroscopy (Model: AA-68000, Shimadzu, Japan) coupled with an auto-sampler (ASC-6100) employing absorption of optical radiation by free atoms in the gaseous state. Metal ion of a sample was quantitatively determined by measuring the radiation absorbed by atoms of the sample solution with respect to a known concentration.

- **FTIR analysis**

FTIR spectroscopy, is an analytical technique used to identify organic, polymeric, and in some cases, inorganic materials. The FTIR analysis method uses infrared light to scan test samples for detecting functional groups and characterizing covalent bonding information. Infrared spectra of the prepared samples were recorded with Shimadju FTIR-8900 (Japan) using KBr disc technique. Pellets were made from 0.2mg CMC samples ground with 2 mg KBr. Transmission was measured at the wave number range of 4000-400 cm^{-1}.

- **Thermal aanalysis**

Thermal analysis is a technique in which a change in the weight of a substance is recorded as a function of temperature or time. Instrument used for thermogravimetry is 'Thermobalance'. Data recorded in the form of a curve known as 'Thermogram'.

Thermogravimetric analyses (TGA) were performed on a Shimadzu TGA-50 system (Japan), with threshold 10 mg samples on a platinum pan, under nitrogen atmosphere. The temperature was increased from the ambient temperature to 600°C at a heating rate of 20°C min^{-1}.

- **X-Ray Diffraction analysis**

X-ray diffraction **(**XRD) is a unique method in determination of crystallinity of a compound. X-ray diffraction peaks are produced by constructive interference of a monochromatic beam of X-rays scattered at specific angles from each set of lattice planes in a sample. The peak intensities are determined by the distribution of atoms within the lattice. Consequently, the X-ray diffraction pattern is the fingerprint of periodic atomic arrangements in a given material.

Diffraction diagrams of samples were recorded using a Bruker D8 Advanced Germany X-Ray Diffractometer that generated CuKα radiation. Powder samples were exposed to X-ray beam (40 kV, 30 mA) at 2°/min.

- **Scanning Electron Microscopy analysis**

The scanning electron microscopy (SEM) was studied to analyze morphology (the granule surface and shape) of a sample. It is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The

electrons interact with atoms in the sample, producing various signals that can be detected and that contain information about the sample's surface topography

Thin layers of cellulose and CMC samples were conditioned in desiccator and coated with gold using an ion sputter under vacuum before analyses. The coated samples were viewed and photographed by the scanning electron microscope (Model-S 3400 N, VP SEM, Hitachi, Japan) using 20 kV accelerating voltage.

- **^{13}C NMR Spectra analysis**

^{13}C NMR is an important tool in chemical structure elucidation in organic chemistry and allows the identification of carbon atoms by giving a single sharp peak for each type of carbon in the molecule.

The prepared CMC film was dissolved in D_2O. Then the samples were examined by ^{13}C NMR spectra with proton decoupling recorded at 25°C on a Bruker, Switzerland NMR spectrometer at a frequency of 400MHz and with 13223 scans. The spectrum range was carried out from 0 to 200 ppm.

- **Microbiological test**

To ensure safety of food products, microbiological tests such as, testing for pathogens and spoilage organisms must be required. Apart from detecting spoilage, microbiological tests can also determine germ content; identify Yeasts and Molds, and *Salmonella*.

The presence of microorganisms such as Yeasts and Molds, *E.coli, Coliform* and *Salmonella* in the prepared CMC were determined following by the method described in the literature [JECFA, 1997]. Detailed procedures are given below:

Sterilization

All tests were carried out in a laminar air flow and all types of precautions were carefully maintained to avoid any contamination during the test. UV light was switched on one h before working in laminar hood to avoid accidental contamination. Petridishes and other glassware were sterilized by autoclave at a temperature of 121°C and pressure of 15 lb/sq.in. for 20 min. Blank discs were kept in a covered petridish and then subjected to dry heat sterilization at 180°C for 1 h. They were then kept in laminar hood under UV light for 30 min.

Total plate count

Using aseptic technique, 1 g of sample was dispersed into 99 ml of phosphate buffer and it was fully dissolved by using shaker or stirrer. Dissolving time was about 10 min. Then 1 ml of the solution was pipetted into separate, duplicate, appropriately marked petridishes. Then 12-15 ml of Plate Count Agar previously tempered to 44-46°C was poured over the aliquot of sample in each petridish, mixed well by alternate rotation, and back and forth motion of the plates, allowed the agar to solidify. The plates were then inverted and finally incubated for 48±2 h at 28±1°C.

After incubation, the growing colonies visible on each plate were counted and recorded the number of colonies. Then the average of both plates was taken and multiplied by the sample dilution factor, 100. Where no colonies are visible, the result is expressed as less than 100 cfu/g.

The Plate Count Agar was used to conduct this experiment and its compositions are given below:

Table 2.1 Composition of Plate Count Agar media.

Ingredient	**Quantity**
Yeast extract	2.5 g
Dextrose	1.0 g
Sodium chloride	5.0 g
Agar powder	9.0 g
Tryptone	5.0 g
Distilled water	1000 ml
Final pH	7±0.2 at 25°C.

E. coli determination

Using aseptic technique, 1 g of sample was dispersed into 99 ml of Lactose broth using either a Stomacher, shaker or stirrer to fully dissolve the sample and kept for 15 min. Then the container was sealed lightly and the broth was incubated for 18-24 h at 28±1°C.

1 ml of the incubate was inoculated by using a sterile pipette into a tube containing 10 ml Gram Negative (GN) broth. It was incubated for 18-24 h and then streak any GN broths showing positive growth or gas production onto duplicate plates of Levine EMB agar. The plates were incubated for 24±2 h at 28±1°C and then examined for colonies typical of *E.coli* i.e. showing strong purple growth with dark centre and a green metallic sheen sometimes spreading onto the agar, then recorded any typical *E.coli* colonies as presumptive positive, otherwise negative.

The composition of Gram Negative broth used in the experiment is given below:

Table 2.2 Composition of Gram Negative (GN) broth media.

Ingredient	**Quantity (g)**
Peptone	20 g
Dextrose	1.0 g
Mannitol	2.0 g
Sodium citrate	5.0 g
Sodium deoxycholate	0.5 g
Potassium phosphate (dibasic)	4.0 g
Potassium phosphate (monobasic)	1.5 g
Sodium chloride	5.0 g
Distilled water	1000 ml
Final pH	7±0.2 at 25°C.

Yeasts and Molds

Using aseptic technique, 1 g of sample was dispersed into 99 ml of phosphate buffer and it was fully dissolved by using shaker or stirrer. Dissolving time was about 10 min. Then 1 ml of the solution was pipetted into separate, duplicate, appropriately marked petridishes. Then 15-20 ml of Potato Dextrose Agar (either acidified or containing antibiotic) previously tempered to 44-46°C was poured over the aliquot of sample in each petridish, mixed well by alternate rotation, and back and forth motion of the plates, allowed the agar to solidify. The plates were then inverted and finally incubated for 5 days at 20-25°C.

After incubation, the growing colonies visible on each plate were counted using a colony counter and recorded the number of colonies. Then yeasts were separated from the molds according to their morphology and counted them separately. The average of both plates was taken and multiplied by the sample dilution factor, 100. Where no colonies are visible, the result is expressed as less than 100 cfu/g.

The Potato Dextrose Agar was used to conduct this experiment and its composition is given below:

Table 2.3 Composition of Potato Dextrose Agar (PDA) media.

Ingredient	**Quantity**
Potatoes (sliced washed unpeeled)	200 g
Dextrose	20 g
Sodium chloride	5.0 g
Agar powder	20 g
Distilled water	1000 ml
Final pH	7±0.2 at 25°C.

Salmonella determination

Using aseptic technique, 5 g of sample was dispersed into 200 ml of sterile Lactose Broth using either a Stomacher, shaker or stirrer to maximize dissolution over a 15 min period. The container was sealed loosely and incubated for 24±2 h at 28±1°C, then examined for colonies typical of *Salmonella* i.e. showing colourless colony and recorded as presumptive positive, otherwise negative.

The Lactose Broth media was used to conduct this experiment and its compositions is given below:

Table 2.4 Composition of Lactose Broth media.

Ingredient	**Quantity**
Peptic digest of animal tissue	5.0 g
Lactose	5.0 g
Beaf extract	3.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml
Final pH	6.9±0.2 at 25°C.

Coliform determination

Using aseptic technique, 5 g of sample was taken into 95 ml of sterile Lactose Broth and incubated for 24-72 h at 25-30°C. When the growth of microorganisms in the tube was observed, it was shaken slightly. Then a portion of the fluid was taken using an inoculating loop, streaked it on MacConkey agar medium, and incubated for 18-24 h at 25-30°C. The plate was examined for suspicious colonies. If red-brick colonies of Gram-negative rods surrounded by a reddish precipitation zone are not found, the sample is determine to be negative.

The composition of MacConkey agar media used in the experiment is given below:

Table 2.5 Composition of MacConkey agar media.

Ingredient	**Quantity**
Peptone (Pancreatic digest of gelatin)	17 g
Proteose peptone (meat and casein)	3 g
Lactose monohydrate	10 g
Bile salts	1.5 g
Sodium chloride	5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Distilled water	1000 ml
Final pH	7.1±0.2 at 25°C.

- **The main instruments used during the studies are given below:**

Forced convection oven

Electronic moisture meter

Muffle furnace

Water bath

NMR Spectrometer

Scanning Electronic Microscope

FTIR Spectrometer

TGA Analyzer

UV-Visible Spectrophotometer

Magnetic Stirrer

Atomic Absorption Spectroscopy

2.3 RESULTS AND DISCUSSION

- **Chemical composition of corn waste**

One of the largest cellulosic agro-industrial by-products is corn waste such as cobs, leaves, husks, stalks etc; fibrous residue left over after the harvesting and processing of corn. An attempt to transform maize husk into CMC, first it is necessary to know the composition of every part of maize waste. As α-cellulose was used as feedstock for the production of CMC so it is essential to analyze, which part contain higher amount of α-cellulose. The chemical composition of the maize waste, mostly cellulose, hemicellulose, lignin, pectic matters, fatty & waxy matters etc. from cobs, husks, leaves and stalks were estimated and is shown in **Table 2.6.**

Table 2.6 Estimation of composition of corn waste (as the average of four replicates).

Name of waste	α – Cellulose (wt%)	β – Cellulose (wt%)	γ- Cellulose (wt%)	Lignin (wt%)	Aqueous extracts (wt%)	Fatty and waxy matters (wt%)	Pectic matters (wt%)	Other (wt%)
Cobs	40.20	17.40	17.10	18.01	2.05	2.00	3.10	0.14
Husks	45.13	16.86	14.29	14.32	2.50	2.20	3.65	1.05
Leaves	40.78	15.61	14.86	17.93	3.00	2.50	3.97	1.35
Stalks	36.51	18.05	17.01	19.30	2.77	2.21	3.59	0.56

The α-cellulose was extracted using the described method and the yield (in g) obtained was very satisfactory per 100 g of dry biomass. From **Table 2.6**, it can be seen that the α-cellulose contents in cob, husk and leaves were higher than 40%, while in stalks, α-cellulose content was lower (i.e. 36.51%). However, the higher α-cellulose content is required for the production of good quality cellulose derivatives. Lower α-cellulose content implies the presence of an excessive amount of low molar

mass oligosaccharides, which may affect the yield and quality of the derivative [Mark *et al.,* 1985].

The highest contents of hemicelluloses (β- and γ-celluloses) and lignin were found in cobs and stalks, while these were lowest in leaves and husks. The amount of fatty and waxy matters was above 2%, in all parts, pectic matters constituted more than 3% of all the waste samples. The aqueous extracts for all the waste samples were in the range of 2-3%. Jeffries [1994] has reported that the composition, within a single plant, varies with age, stage of growth, and other conditions. As we are particularly interested for α-cellulose and husk contain higher percentages of α-cellulose, so maize husk was used as source material for producing CMC.

- **Characterization of raw husk powder and purified extracted cellulose by FTIR**

Characterization of extracted cellulose was done by FTIR spectroscopy. The differences between the raw corn husk powder and the extracted cellulose powder can be seen in the FTIR spectra presented in **Figure 2.1**. The major peaks were tentatively assigned and the data were compared with those found in the literature tabulated in **Table 2.7** [Colom *et al.,* 2003; Adebajo and Frost, 2004]. The main alterations were found in the region from 1800 to 1000 cm^{-1}. The absorption peaks at 1740.13, 1638.49, 1605.18 and 1514.15 cm^{-1} were not observed in the extracted cellulose.

Particularly, two absorption peaks must be emphasized: the peaks at 1514 and 1249 cm^{-1}. The peak at 1514 cm^{-1} was not present and the peak at 1249 cm^{-1} was drastically reduced on the purified cellulose spectra. These two absorption peaks were important because their absence in the extracted

cellulose spectra strongly indicates that most of the lignin had been removed [Kondo, 1997 and Ivanova *et al.,* 1989].

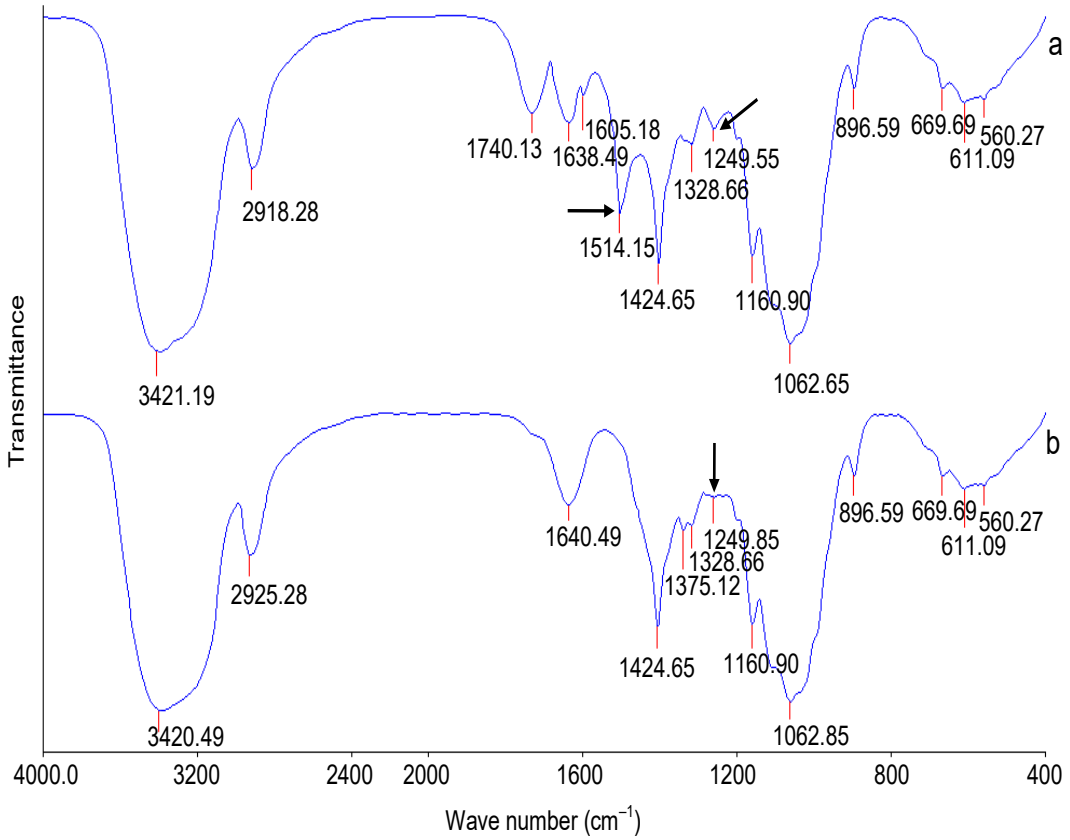


Figure 2.1 FTIR spectra of a) raw corn husk powder and b) purified extracted cellulose

Table 2.7 Assignment of main absorption peaks in raw and extracted cellulosic materials.

Wave number (cm^{-1})		Assignment
Raw corn husk powder	**Extracted cellulose powder**	
3421.19	3420.49	OH stretching
2918.28	2925.28	CH stretching of
1740.13	---	C=O stretching of acetyl or carboxylic acid
---	1640.49	H-O-H bending of absorbed water
1638.49	---	Carbonyl stretching with aromatic ring
1605.18	---	C=C stretching of aromatic ring (lignin)
1514.15	---	C=C stretching of aromatic ring (lignin)
1424.65	1424.65	CH_2 bending
---	1375.12	C-H deformation
1328.66	1328.66	OH in plane bending
1249.55	1249.85	C-O stretching of ether linkage
1160.90	1160.90	C-O-C antisymmetric bridge stretching
1062.85	1062.85	C-O symmetric stretching of primary alcohol
896.59	896.59	β- glucosidic linkages between the sugar units

- **Determination of the optimized reaction conditions**

Carboxymethylation reaction of α-cellulose isolated from corn husks was optimized, with respect to DS by varying cellulose particle size and other reaction parameters to get higher DS. CMC is characterized in terms of DS because the industrial utility of the CMC largely depends on DS. Higher the DS, higher the product quality, purity and yield. This test method is used to determine the number of substituent groups added to the basic cellulose backbone. The most common DS obtained from commercial CMC is usually lower, from 0.4 to 1.4 [Durcilene *et al.,* 2004]. Researchers have tried to get higher DS through carboxymethylation. So, all the optimization experiments were done with respect to the DS.

Effect of cellulose particle size on DS

Particle size plays a great role in determining DS for the preparation of CMC. The reactions were carried out at five different cellulose particle sizes such as 1071, 340, 149, 100 and 74 µm. The effect of particle size on DS was studied as given in **Figure 2.2.**

The reaction time was kept at 3.5 h and temperature was 55°C. **Figure 2.2** show that DS increases with decrease of particle size. The carboxymethylation mainly depends upon the accessibility of reactants and the availability of the activated hydroxyl groups. When particle size decreases, surface area as well as available free -OH groups for substitution reaction increases, thus DS increases.

Reduced cellulose particle size has larger surface area per unit volume and, therefore more cellulose may be accessible for the reactants to reach and at a faster rate [Millett *et al.,* 1976; Fan *et al.,* 1982].

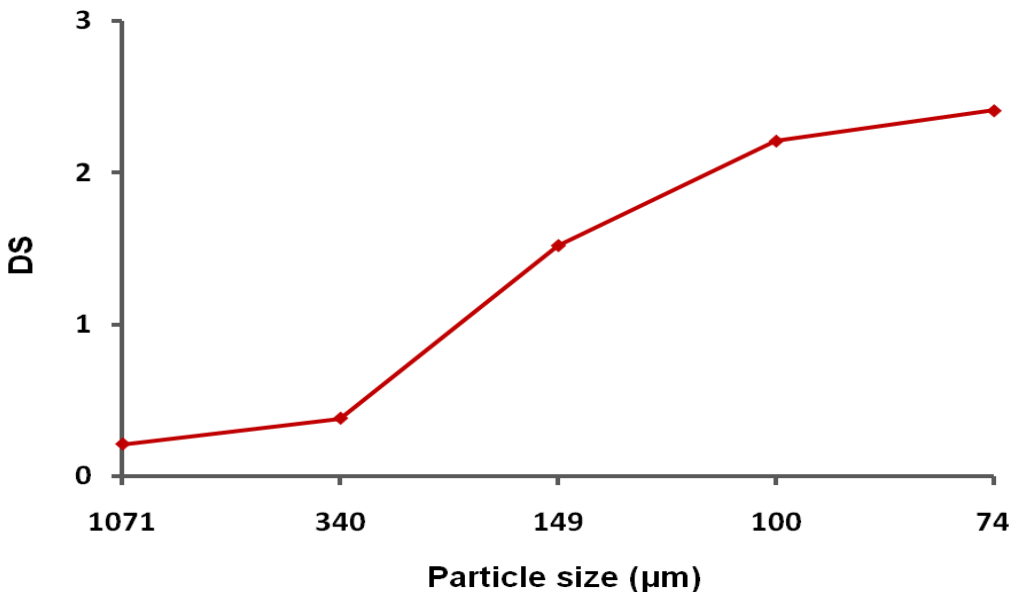


Figure 2.2 Effect of cellulose particle size on DS (NaOH, 30%; MCA, 12.69 mol/L; temp., 55°C; time 3.5 h).

Effect of sodium hydroxide concentration on DS

The sodium hydroxide (NaOH) concentration plays an important role in the production of CMC and by-products. The carboxymethylation process was carried out at different concentrations of NaOH ranging from 20 to 40% (w/v), while other parameters were kept constant. The DS value was found to increase significantly when the concentration of NaOH increased to 30% (w/v), thereafter it decreased considerably, as shown in **Figure 2.3**.

The higher concentration of alkali is not preferable as a higher concentration of sodium hydroxide reacts with monochloroacetic acid to form sodium glycolate,

according to the reaction 1, resulting in the inactivation of the monochloroacetic acid [Khaullar *et al.,* 2005].

Main reaction:

$R_{cell}OH + ClCH_2COOH + NaOH = R_{cell}OCH_2COONa + NaCl + H_2O$

Side reaction:

$NaOH + ClCH_2COOH = HOCH_2COONa + NaCl$……1
(Sodium glycolate)

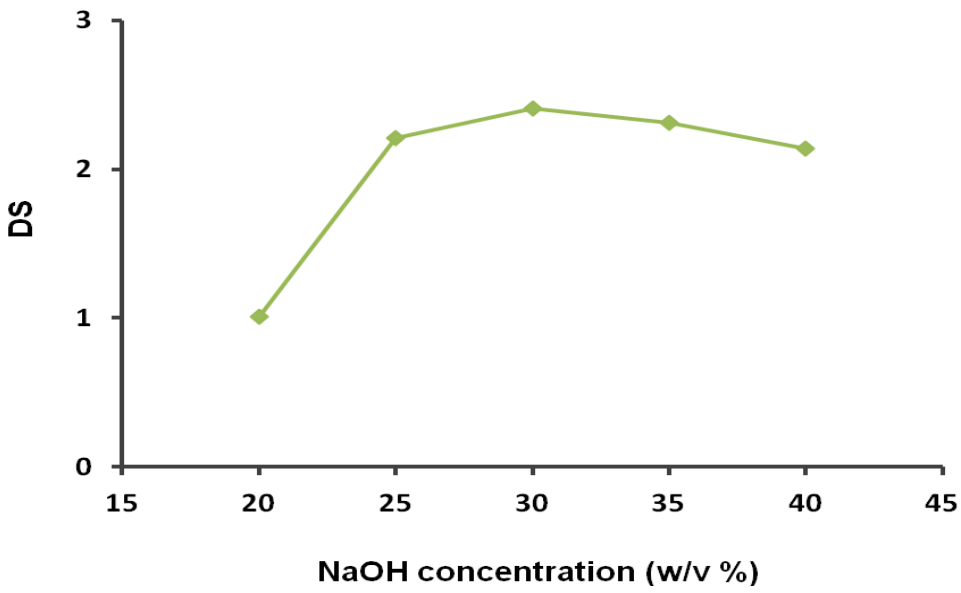


Figure 2.3 Effect of NaOH concentration on DS (MCA, 12.69 mol/L; temp., 55°C; time, 3.5 h).

Effect of monochloroacetic acid concentration on DS

The proportion of monochloroacetic acid (MCA) concentration and NaOH concentration must be at optimum to obtain a high DS value. **Figure 2.4** illustrates the effect of MCA concentration on DS. A maximum DS of 2.41 was obtained with

12.69 mol/L of MCA concentration. The increase is probably due to the greater availability of the acetate ions at higher concentrations in the proximity of cellulose molecules.

At a concentration higher than 12.69 mole/L MCA, glycolate formation seems to be favored and the reaction efficiency decreased. This finding is supported by the reports of Khalil *et al.* [1990] and Bhattacharyya *et al.* [1995].

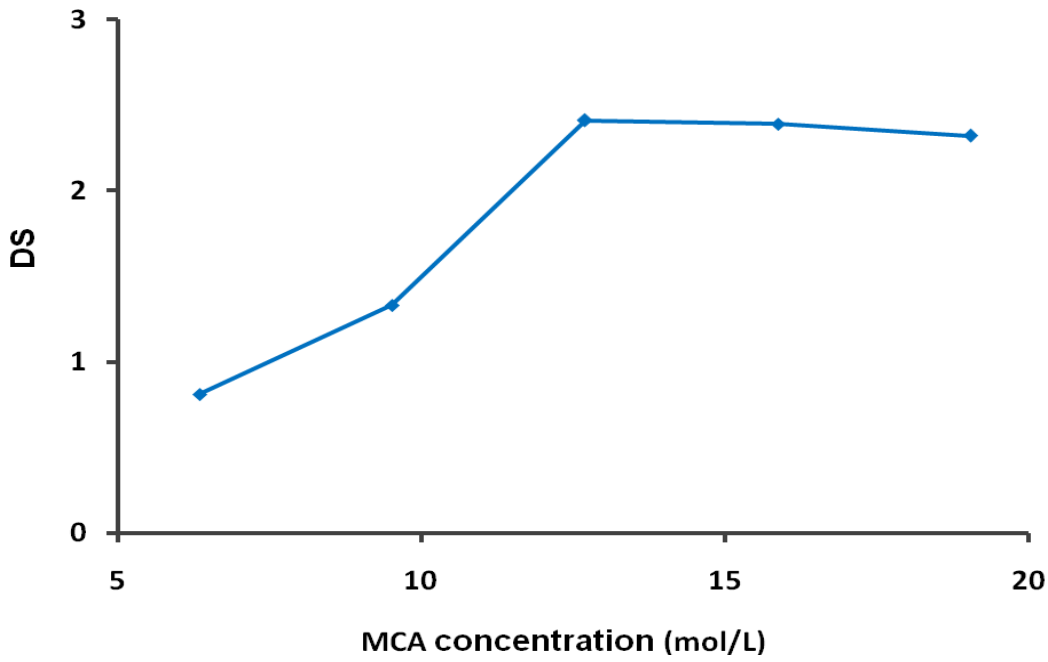


Figure 2.4 Effect of MCA concentration on DS (NaOH, 30% (w/v); temp., 55°C; time, 3.5 h).

Effect of time on DS

As shown in **Figure 2.5**, the carboxymethylation reaction was carried out in six different reaction periods. The maximum DS value attained was 2.41 at 3.5 h. Further increase in time resulted in a lowering of DS. The increase in DS value at longer reaction times was due to the effect on the swelling ability of the cellulose

molecules, as well as the diffusion and absorption processes of the reactants, with improved contacts between the etherifying agents and cellulose [Khalil *et al.,* 1990].

The lowering of DS after 3.5 h of carboxymethylation time may also be due to the atmospheric oxidative degradation of CMC. Similar influence of reaction duration on DS of carboxymethylated flax cellulose has been reported by Hebeish *et al.* [1984].

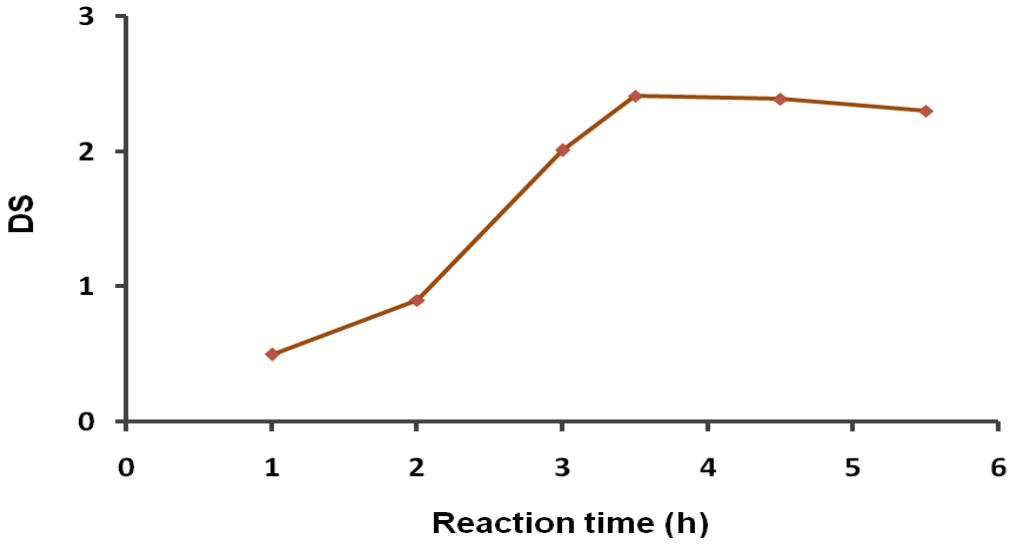


Figure 2.5 Effect of reaction time on DS (NaOH, 30%; MCA, 12.69 mol/L; temp., 55°C).

Effect of temperature on DS

The influence of the reaction temperature on the DS is shown in **Figure 2.6**. The temperature for the carboxymethylation process was varied from 35°C to 70°C. The DS value increased with the increase in reaction temperature approaching its maximum point at 55°C. The maximum value of DS achieved was 2.41.

By increasing the temperature, the diffusion of MCA increased more readily to a reaction point which created more favourable environment for the reaction to take place [Stojanovic *et al.,* 2000]. On the other hand, the values of DS decreased sharply by further increment in the reaction temperature from 55°C to 70°C. Lowering of the DS beyond 55ºC could be attributed to the degradation of carboxymethylated cellulose under the influence of atmospheric oxygen. Similar observations have been observed for cellulose carboxymethylation in literature too [Hebeish *et al.,* 1984; Tijsen *et al.,* 2001; Youssef *et al.,* 1989].

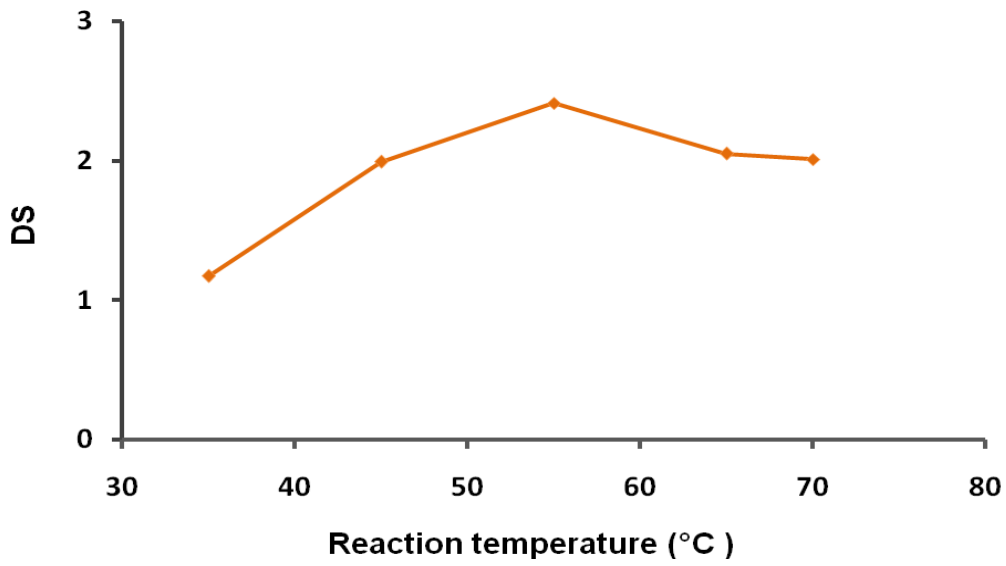


Figure 2.6 Effect of reaction temperature on DS (NaOH, 30%; MCA, 12.69 mol/L; time, 3.5 h).

➢ **Preparation of CMC from corn husk cellulose**

The cellulose powder of different particle size from corn husk was modified by carboxymethylation reaction. **Scheme 1** summarized the key steps in producing CMC from the α-cellulose extracted from corn husk. The method of this process is

the reuse of alcohol recovered through distillation, which makes the process economic.

CMC was prepared from corn husks cellulose at different cellulose particle sizes under optimum time, temperature, NaOH and monochloroacetic acid concentration. During the reaction process, the recovered ethanol obtained by CMC preparation process through distillation was above 90% which was reused. The yield of CMC at different cellulose particle sizes is shown in **Table 2.8.** It can be observed from the Table that % yield of CMC increases with decreasing particle size and highest yield obtained is 240.58% with respect to particle size 74 µm. Reduced cellulose particle sizes have larger surface area, so excess reactants can penetrate into the cellulose at a time.

Sharma and Sharma [1994] reported that decreased cellulose particle size provides a greater surface area which increases the chance of collisions between reactants and cellulose particles, so the reaction rate increases as well as the production of CMC increases.

The yield of CMC product in this work was very high, 2.40g/g. According to Silva *et al.* [2004], the yield is certainly a function of the amount of material lost during dialysis step. More degradation occurred and larger amount of low molecular weight material were released when more drastic reaction conditions (higher temperature, NaOH and SMCA concentration) is applied. This will results in smaller amount of carboxymethylated polymeric product.

Table 2.8 Preparation of CMC from corn husk cellulose in aqueous ethanolic medium with different cellulose particle size (Temperature 55°C; Time 3.5 h).

Particle size (µm)	Weight of the sample (g)	Amount of NaOH (mol/L)	Amount of $ClCH_2COOH$ (mol/L)	Yield of CMC (%)
1071	5	7.5	12.69	100.89
340	5	7.5	12.69	115.75
149	5	7.5	12.69	195.51
100	5	7.5	12.69	210.87
74	5	7.5	12.69	240.58

- **Determination of degree of substitution**

Degree of substitution (DS) represents the number of the carboxymethyl groups in the molecular unit of the anhydroglucose units. In principle, all hydroxyl groups (HO-2, HO-3, and HO-6) in the anhydroglucose unit can be substituted, and the maximum DS is being 3 [Salmi *et al.,* 1994]. DS is the most important factor because CMC is available in several grades and in a variety of types depending on the degree of substitution [Mark *et al.,* 1985]. A number of methods are available for determining the DS, e.g chemical method, ASTM D1439, IR, Raman, NMR and SEM-EDX [Singh and Khatri, 2012] etc. Here, we have used the chemical method where DS was chemically determined as the average of three replicate analyses.

DS of the synthesized CMC was determined in terms of cellulose particle size and solubility was also tested in water given in **Table 2.9**.

Table 2.9 Determination of degree of substitution (DS) of CMC synthesized from corn husk cellulose at different particle sizes.

Particle size (µm)	Weight of sample (g)	Weight of ash (%)	Quantity of H_2SO_4 (0.1N) required for titrating ash (ml)	Degree of substitution	Solubility
1071	2	13.01	23.30	0.21	Insoluble in water
340	2	13.19	39.30	0.38	Insoluble in water
149	2	17.94	106.98	1.52	Highly soluble in water
100	2	18.23	130.60	2.21	Highly soluble in water
74	2	18.81	135.10	2.41	Highly soluble in water

From **Table 2.9**, it can be seen that the DS of the prepared CMC increase gradually with the decrease of cellulose particle sizes and highest DS obtained is 2.41 with respect to particle size 74 µm. Reduced cellulose particle sizes have larger surface area, so excess reactants can penetrate into the cellulose at a time. The etherification mainly depends upon the accessibility of reactants and the availability of the activated hydroxyl groups [Alam and Mondal, 2013]. When particle size decreases, surface area as well as number of available free –OH groups for substitution reaction increases, thus DS increases. Reduction in particle size of cellulose could enhance the affinity between cellulose particles and reactants and thus increases the etherification rate as well as the carboxymethyl substitution rate [Yeh *et al.,* 2010].

The solubility of the prepared CMC samples with different particle sizes as well as DS was observed and it was found that CMC with DS 0.21 and 0.38 were insoluble in water but CMC with DS 1.52 to 2.41 were highly soluble in water. CMC with DS less than 0.40 is insoluble but above this value of DS, CMC is fully soluble with its hydro affinity increasing with increasing DS [Varshney *et al.,* 2006].

A comparison between DS value of CMC prepared from corn husk cellulose and CMC from other sources cellulose is presented in **Table 2.10**. The DS values of reference CMC were almost the same, but the DS value in this method was very high, 2.41 compared to reference values.

Table 2.10 DS value of CMC from different agro cellulosic source.

Sources of cellulose	**Reference**	**Degree of substitution**
Water hyacinth	Barai *et al.,*1997	0.24-0.73
Sago waste	Pushpamalar *et al.,* 2006	0.33-0.82
Sugar beet pulp cellulose	Togrul and Arslan, 2003	0.11-0.67
Lantana camara	Varshney *et al.,* 2006	0.20-1.22
Palm kernel cake	Bono *et al.,* 2009	0.67
Durian husk	Siralertmukul *et al.,* 2005	0.67
Corn husk	This work	2.41

- **Determination of intrinsic viscosity of synthesized CMC**

Intrinsic viscosity is a widely used measure of the polymers molecular weight and is used as part of the specification to select the right grade of CMC for a particular application [http://www.ametektest.com/learningzone/library/articles/the-importance-of-intrinsic-viscosity measurement. SULLC029.dpuf]. The determination

of the solution viscosity of polymeric materials is very important to the industry, both to research and manufacturing, since it can be used to estimate molar mass providing important information relating to the physical properties of polymers.

Intrinsic viscosity was determined from the plot of reduced viscosity (η_{red}) versus concentration as given in **Figure 2.7**. The intercept of the plots on the ordinate at c = 0 gives intrinsic viscosity [η].

Figure 2.7 shows that the intrinsic viscosity of CMC increased gradually with the reduced of cellulose size which is also shown graphically in **Figure 2.8**. This is due to the increase in higher conversion of cellulose into CMC. As cellulose particle size decreases, surface area where the reaction takes place increases. Reduced α-cellulose particle size provides a greater surface area, which increases the chance of interactions between reactant and α-cellulose. This allows the reaction rate to increase, as well as the yield and higher conversion of CMC [Sharma and Sharma, 1984].

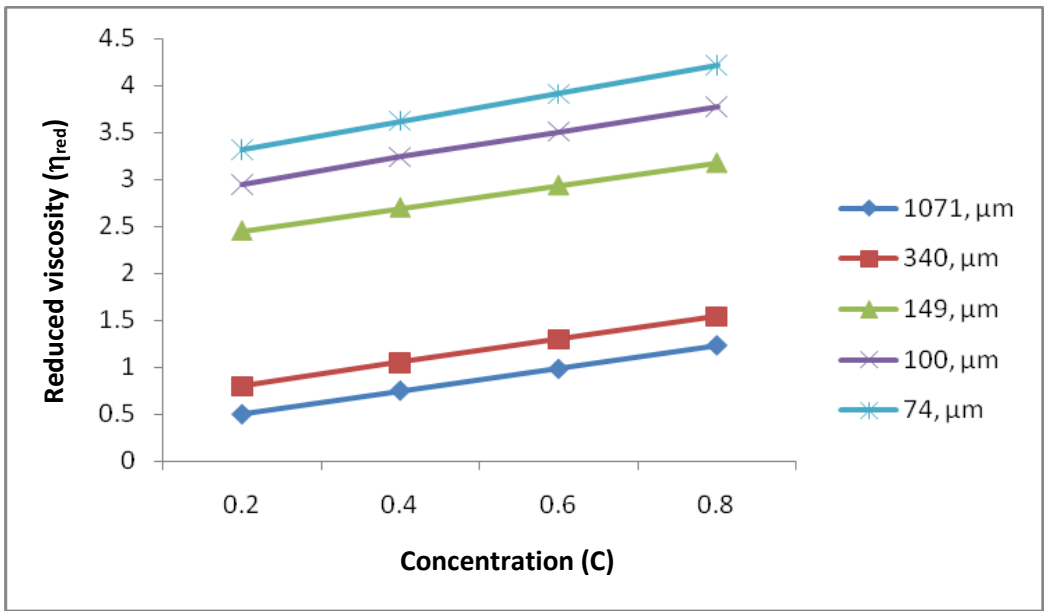


Figure 2.7 Plot of reduced viscosity vs. concentration

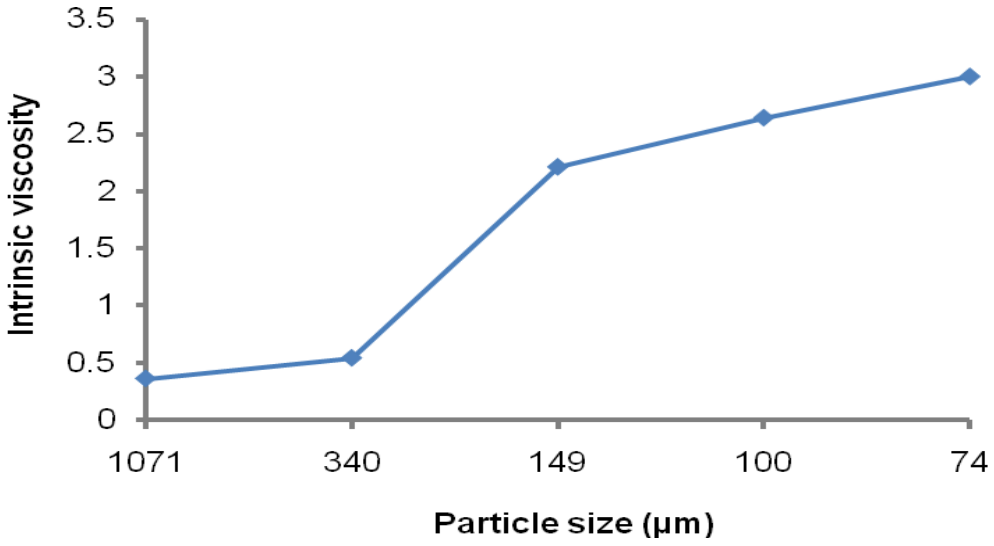


Figure 2.8 Graphical presentation of particle size and intrinsic viscosity.

- **Determination of molecular weight of CMC**

The most important application of viscosity in the field of polymer chemistry is in the determination of molecular weight. High molecular weight polymer has high viscosity. The determination of the molecular weight of polymeric materials is very important to the industry because it determines the physical and mechanical properties of a polymer.

It is reported by Mark *et al.* [1985] that the ability of CMC to function as a thickener or flow-control agent depends largely on its degree of substitution and on the molecular weight. The molecular weight of the CMC prepared from different cellulose particle size was determined and the data presented in **Table 2.11**.

Table 2.11 Determination of molecular weight of prepared CMC as a function of particle size (molecular weight was calculated from $[\eta]=KM^a$ equation, Value of K = 37× 10^{-5} dl/g and a = 0.61 at 35°C).

Particle size (µm)	Degree of substitution	Intrinsic viscosity [η]	Molecular weight (Da)
1071	0.21	0.36	79522
340	0.38	0.54	154623
149	1.52	2.21	1559381
100	2.21	2.64	2087277
74	2.41	3.00	2574111

It can be seen from the **Table 2.11** that the molecular weight of the prepared CMC increased with the decrease of cellulose particle size. Such molecular weight also increased with the increase of DS. As the DS increased, the number of OH groups was replaced by carboxymethyl groups. As the carboxymethyl group is heavier than OH group, the molecular weight of the final product CMC increased [Alam and Mondal, 2013].

There is a great relationship between intrinsic viscosity and molecular weight shown as graphically in **Figure 2.9**. Figure shows that molecular weight increases with the increase of intrinsic viscosity. Viscosity of a polymer solution depends mainly on the concentration and size (i.e., molecular weight) of the dissolved polymer. Thus more the intrinsic viscosity, larger the polymer size as well as higher the molecular weight [Mondal *et al.*, 2015].

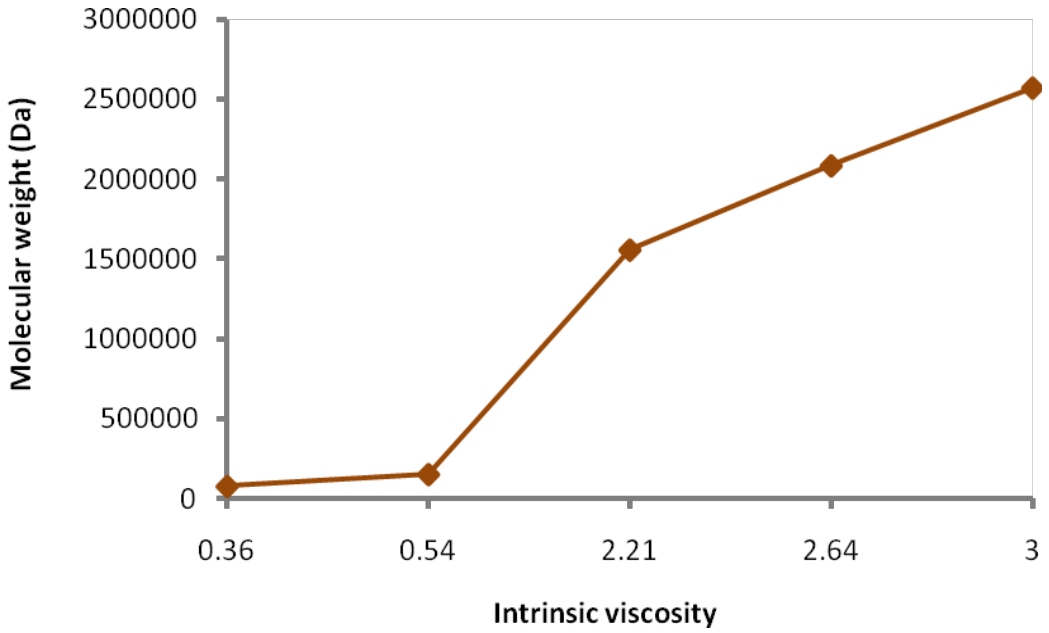


Figure 2.9 Relationship between molecular weight and intrinsic viscosity of prepared CMC

There is a direct relationship between intrinsic viscosity and degree of substitution (DS) of the prepared CMC. Intrinsic viscosity increases with the increase of DS as presented in **Figure 2.10**. Increasing DS means that each anhydroglucose unit (AGU) of constituent reacts with carboxymethyl, thereby increasing the weight of the produced carboxymethyl cellulose. The effect of DS on viscosity was due to the more carboxymethyl groups substituted by the hydroxyl groups of the cellulose molecules. The molecular weight of the anhydrous glucose unit is 162 and 58 is the net increment in the anhydrous glucose unit for every substituted carboxymethyl group.

These carboxymethyl groups act as hydrophilic group, therefore an increase of DS thus increase the ability of CMC to immobilize water in a system [Latif *et al*., 2007].

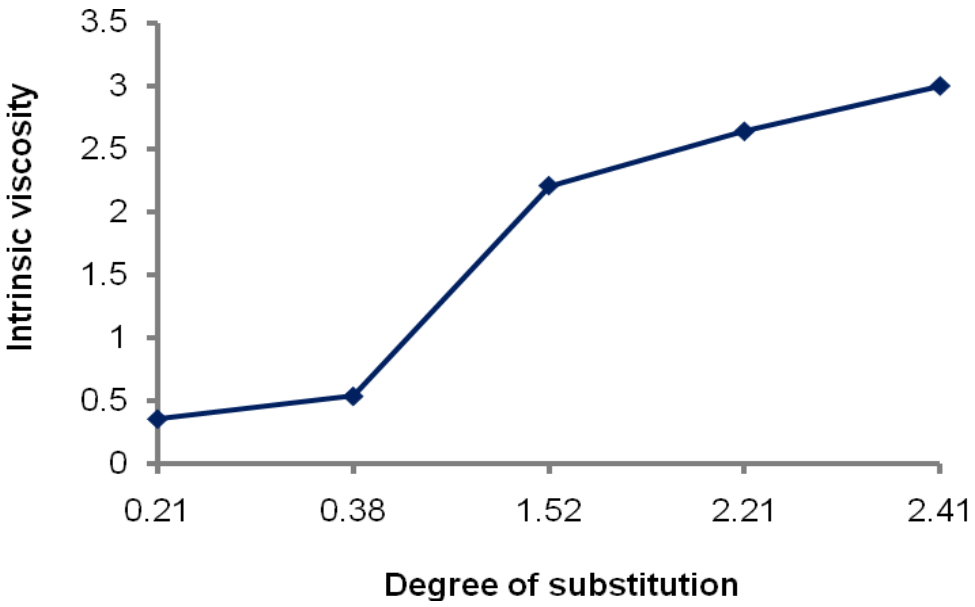


Figure 2.10 Relationship between degree of substitution and intrinsic viscosity of prepared CMC.

- **Toxic metal content in the prepared CMC**

Heavy metals are potential environmental contaminants with the capability of causing human health problems due to their toxic effects even at very low concentrations [Das, 1990].

Metal toxicity is the toxic effect of certain metals in certain forms and doses on life. The main threats to human health from heavy metals are associated with exposure to lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg). These metals have been extensively studied and their effects on human health regularly reviewed by international bodies such as the WHO [Jarup, 2003]. These heavy metals are called toxic metals [Morgan, 1999]. Mankind today is exposed to the highest levels of

these metals in recorded history [http://drlwilson.com/articles/toxicmetals.htm, 18.12.2015].

Toxic metal content in the prepared CMC was determined and compared with the recommended values shown in **Table 2.12**. From the results in **Table 2.12**, it can be observed that the concentrations of Pb, Cd, Hg and As are 0.0001, 0.0005, 0.001 and 0.0002 ppm respectively. These values were within the World Health Organization (WHO) permissible limits for food additives and emulsifier. Both the *Food Chemicals Codex* and the Food and Agriculture Organization of the United Nations World Health Organization (FAO/WHO) have established specifications for identity and purity of CMC which are also met by our prepared food-grade CMC.

Table 2.12 Concentration of heavy metals in the synthesized CMC.

Heavy metal	Concentration in ppm (studied values)	Concentration in ppm (proposed general limit) [JECFA, 1967; JECFA, 2000]
As	0.0001	3
Pb	0.0005	1 or lower in case of high consumption
Cd	0.001	1
Hg	0.0002	1

- **Purity measurement of prepared CMC**

The purity of the CMC was measured after washing the product several times with 70% ethanol and then absolute ethanol which removes the reaction by-products such as sodium chloride and sodium glycolate. It must be check the purity of carboxymethyl cellulose required by public health regulations. Where the CMC

product is intended for human consumption, CMC must be purified to a level of 99.5 percent [US International Trade Commission, 2005].

Table 2.13 shows the different grade of CMC with their applications and also shows that only extra purified CMC is used as food additive. The percentages of CMC content and sodium salts in the prepared CMC were determined and compared to that of standard CMC presented in **Table 2.14**.

It can be observed from the **Table 2.14** that percentages of sodium chloride and sodium glycolate in the prepared CMC are very low, 0.009% and 0.001% respectively. Lower salts content means higher purity. Sodium glycolate is toxic in nature. It is reported from the literature [JECFA, 2002; JECFA, 1997b] that sodium chloride and sodium glycolate contents in the food additives should not more than 0.5% each or in combination.

The purity of prepared CMC was higher, 99.99%, well above the purity of 99.5% for standard CMC and 98% required for commercial grade CMC. Our prepared purified CMC met standard set by the U.S. Code of Federal Regulations, Title 21, Section 182.1745 – Substances that are generally recognized as safe (GRAS).

The purity of CMC increased suggests that, during carboymethylation, more MCA molecules are substituted to the cellulose polymer, thus decreasing the possibility of MCA to react with NaOH to form by-products [Togrul and Arslan, 2003]. The use of alcohol as solvent in the carboxymethylation reaction provides miscibility and accessibility of the etherifying reagent to the reaction center of the α-cellulose chain, rather than glycolate formation [Pushpamalar *et al.,* 2006].

Table 2.13 CMC grades and typical applications [Stigsson *et al*. 2001].

Quality of CMC	Examples of application areas	Content of CMC (%)	Content of salts (%)
Technical	Detergents, mining flotation	< 75	> 25
Semi-purified	Oil and gas drilling muds	75 – 85	15 – 25
Purified	Paper coating, textile sizing and printing, ceramic glazing, oil drilling muds	> 98	< 2
Extra purified (Cellulose gum)	Food, toothpaste, pharmaceuticals	> 99.5	< 0.5

Table 2.14 Determination of CMC, NaCl and sodium glycolate contents.

Parameters	Content of CMC or purity (%)	Content of NaCl (%)	Content of sodium glycolate (%)
CMC prepared	99.99±0.002	0.009±0.06	0.001±0.01
CMC standard	99.95±0.06	0.03±0.07	0.02±0.01

Results are expressed as mean ± standard deviation; n=3

- **Some distinct properties of prepared CMC**

Some distinct properties of the synthesized CMC at DS 2.41 were investigated and the results are shown in **Table 2.15** and **Table 2.16**.

The results of moisture content, ash content, gel content, WHC and OHC of standard CMC and that of prepared CMC are listed in **Table 2.15**. From the Table, it can be seen that moisture content and OHC of standard CMC and prepared CMC are almost similar; but ash content, gel content and WHC of prepared CMC are higher than that of standard CMC. The high WHC proves that prepared CMC are highly hydrophilic because during etherification, more hydroxyl groups were replaced by

carboxymethyl groups that are hydrophilic [Latif *et al.,* 2007]. The high gel content means the high reactivity (Lin *et al.,* 2005). The high ash content implies high DS, perhaps because more hydroxyl groups are substituted by sodium salts of carboxymethyl groups during the etherification reaction [Mondal *et al.,* 2015].

Table 2.16 represents the some physicochemical parameters of prepared CMC obtained from corn husk. Tested results in **Table 2.16** show that produced CMC is white in colour, fine powdered, odourless, tasteless, freely soluble in water and insoluble in ethanol. pH of 1% CMC solution was 6.80. No layer of foam was appeared by shaking 0.1% solution of the CMC sample. This test distinguishes CMC from other cellulose ethers and from alginates and natural gums [International oenological codex, 2012].

No blue or reddish brown colour of CMC was developed with iodine solution and red colour with acidified Phlorogucinol. These tests confirm the absence of starch, dextrin and organic impurities in the prepared CMC. The produced CMC was able to form film. This property makes the CMC suitable for used as drug delivery devices. CMC with rapid swelling properties makes the product suitable for application as a disintegrant pharmaceutical excipient [Bhandari, 2012].

Joint FAO/WHO Expert Committee on Food Additives [1997b] has established some standard for the identification and purity of CMC. The prepared CMC in this study meets these requirements.

Table 2.15 Determination of water holding capacity (WHC), oil holding capacity (OHC); moisture, ash and gel contents of synthesized CMC (DS=2.41) and standard CMC (DS=0.8).

Sample name	Moisture content (%)	Ash content (%)	WHC (g/g)	OHC (g/g)	Gel content (%)
Standard CMC	2.22±0.76	14.23±0.27	4.12±0.18	1.58±0.22	99.06±0.60
Prepared CMC	2.21±0.13	18.05±0.40	5.11±0.24	1.59±0.07	99.96±0.02

Results are expressed as mean ± standard deviation; n=3

Table 2.16 CMC product parameters.

Tests	CMC
Organoleptic	Odourless, white and tasteless with free flowing powder
Solubility	Forms highly viscous solution with water but insoluble in ethanol
Foam	No layer of foam appears by shaking 0.1% solution
pH in 1% solution	6.80
Starch and Dextrin's	No blue or reddish brown colour with iodine solution
Organic impurities	No red colour with acidified Phlorogucinol
Film formability	Able to form film
Swelling ability	Rapid swelling in water

- **Microbiological test**

Microbiological test is very important to understand the microorganisms and their relation to the food industry in terms of food spoilage, food-borne illness, or food-related intoxication. It is essential to detect microorganisms, in particular pathogenic microorganisms in the areas of the food industry to confirm the authenticity of foods.

Yeasts can play an important role in the spoilage of food, some Molds can also produce mycotoxins that can be harmful to humans. *E.coli* can cause food poisoning. They are members of the Enterobacteriaceae group used in food testing as faecal hygiene indicators. *Coliforms* are also used as hygiene indicator organisms. *Salmonella* food poisoning has long been, and continues to be, an important global public health problem. The presence of such microorganisms as Yeasts and Molds, *E.coli*, *Coliform* and *Salmonella* in the prepared CMC were tested and the cultural responses of tested organisms are given in **Table 2.17**.

Table 2.17 Microbiological test of the prepared CMC.

Tested organisms	**Cultural response**
Total plate count, cfu/g	<100
Mold, cfu/g	<100
Yeast, cfu/g	<100
Colliform/g	Negative
Salmonella/ g	Negative
E. coli/ g	Negative

The *total plate count* is intended to indicate the level of microorganisms in a product. This is a widely used test on food which will give an overall picture of microbiological quality.

No colonies were observed in sample containing cultured media after 48±2 h incubation at 28±1°C. Where no colonies are visible, the result is expressed as less than 100 cfu/g [JECFA, 1997a].

Yeasts grow as creamy to white colonies and Molds grow as filamentous colonies of various colours on Potato Dextrose Agar (PDA) media. When 1 g sample aliquot was poured in a plate containing PDA, no colonies were visible after 5 days incubation of plate at 20-25°C that confirms the absence of Yeasts and Molds in the sample.

E.coli grows as dark blue to violet colonies*, coliform as* salmon to red colonies and *Salmonella* as colourless colony. Results in **Table 8** also show that *Coliform, Salmonella* and *E.coli* either failed to grow or produced negative results with their respective culture media.

- **FTIR analysis**

The infrared spectra were studied to confirm the substitution reaction in carboxymethylation. The FTIR spectra of all prepared CMC samples, with different DS, are shown in **Figure 2.11**. It is evident from **Figure 2.11** that the broad absorption peak at 3434.92 cm^{-1} is due to the stretching frequency of the –OH group. A peak at 2924.07 cm^{-1} is attributable to C-H stretching vibration.

The presence of a new and strong absorption peak at 1620.16 cm^{-1} confirms the stretching vibration of the carboxyl group (COO^-), 1423.81 cm^{-1} is assigned to

carboxyl groups as the sample salts [Bono *et al*., 2009; Biswal and Sing, 2004]. It is an evidence that hydroxyl group of cellulose was replaced with carboxyl group when carboxymethylation reaction occur. Mario *et al*. [2005] have found that the carboxyl groups and their salts wave numbers are between 1600-1640 cm^{-1} and 1400-1450 cm^{-1}, respectively. The peaks at 1329.32 cm^{-1} and 1112.65 cm^{-1} are assigned to -OH bending vibration and –C-O-C stretching, respectively. Wavelength 894 cm^{-1} is detected for 1, 4-β glycoside of cellulose [Viera *et al.,* 2007].

The IR spectra of the standard CMC procured from the market are also recorded in **Figure 2.11a**. Quite similar IR spectra are present with a slight variation in position, like the >CH-O-CH_2 stretching peak at 1061.04 cm^{-1}. Here, the spectra of the synthesized CMC with higher DS show distinctly higher intensity than in the spectra of the CMC with comparatively lower DS, including commercial CMC in **Figure 2.11**. Similar result was observed by the report of Helene *et al*. [2013].

FTIR spectral data of standard CMC and prepared CMC are listed in **Table 2.18**.

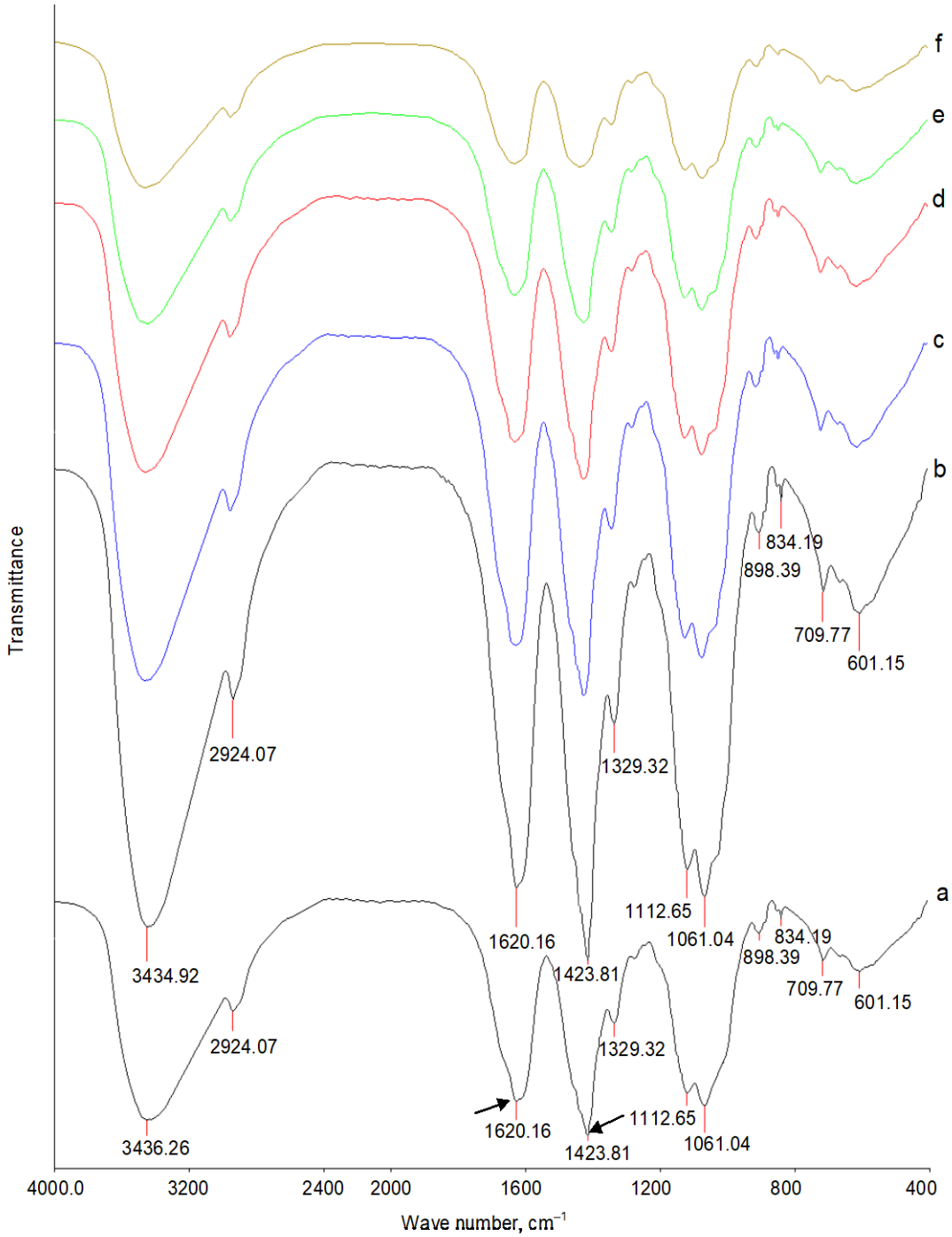


Figure 2.11 FTIR spectra of standard and prepared CMC with different DS, (a) standard CMC (DS=0.8), (b) CMC with DS 2.41, (c) CMC with DS 2.21, (d) CMC with DS 1.52, (e) CMC with DS 0.38, and (f) CMC with DS 0.21.

Table 2.18 Assignment of main absorption peaks in carboxymethyl cellulose.

Wave number (cm^{-1})		Assignment
Standard CMC	Prepared CMC	
3436.26	3434.92	OH stretching
2924.07	2924.07	CH stretching CH_2 and CH_3 groups
1620.16	1620.16	C=O region (indicated CMC)
1423.81	1423.81	CH_2 bonding (indicated CMC)
1329.32	1329.32	OH in plane bending
1112.65	1112.65	C-O-C asymmetry bridge stretching
1066.44	1061.04	C-O symmetry stretching alcohol
898.39	898.39	-β glycoside linkage

- **XRD analysis**

X-Ray diffraction (XRD) analysis is a definitive technique for estimating the degree of crystallinity in polymer. Cellulose is semi-crystalline in nature which. **Figure 2.12** shows diffraction pattern of (a) pure husk cellulose and (b) carboxymethylated cellulose. The peaks correspond to the crystalline phase and the background corresponds to the amorphous phase. The peaks are broad due to the small crystallites in cellulose granules, which is in agreement with the theory of XRD. This theory state that very small imperfect crystals give broadened diffractions.

Crystallinity of the extracted celluloses is 64.39%. The diffraction spectra of CMC show a destruction of the crystalline structure of the original cellulose. All characteristic peaks for native cellulose have almost disappeared and transformed into an amorphous phase. Therefore CMC has excellent solubility [Zhang *et al.,* 1992] as lower crystallinity represents higher solubility [He *et al.,* 2009]. During the carboxymethylation process, the cellulose molecules are placed in alkaline solution.

The swelling of the cellulose granules exert a tension on neighbouring crystalline of cellulose molecules and tend to distort them. Further swelling leads to uncoiling or dissociation of doubled-helical region and the breakup of crystalline structure [Fang *et al.,* 2002].

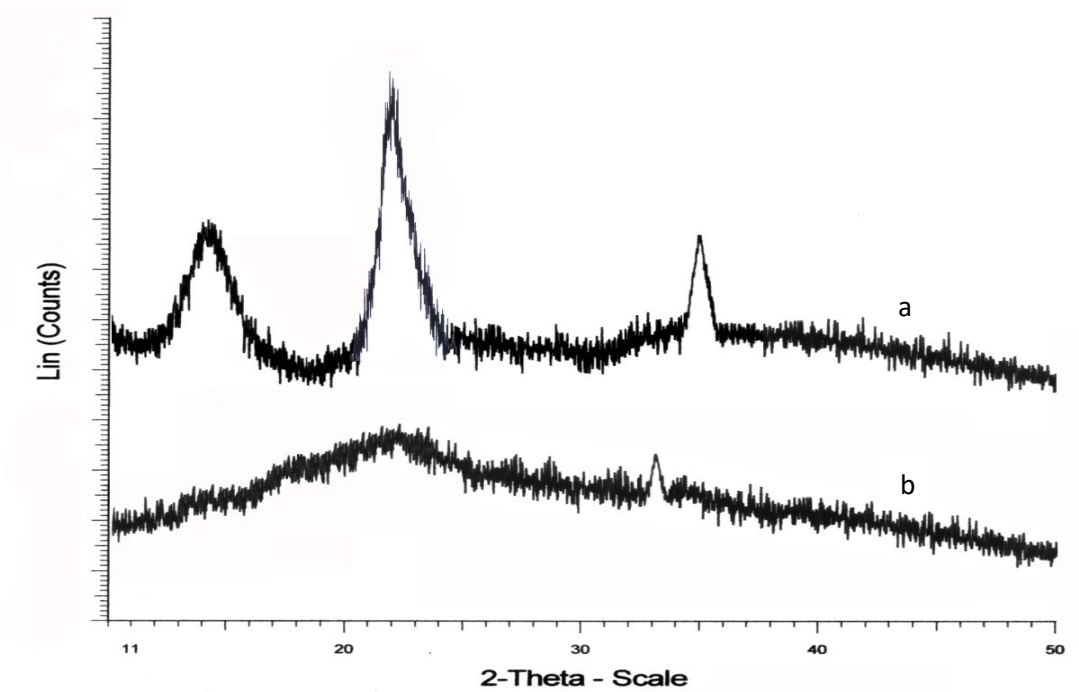


Figure 2.12 X-ray difractogram of (a) pure husk cellulose and (b) carboxymethylated cellulose (DS=2.41).

The X-Ray diffraction patterns of synthesized carboxymethyl celluloses with different particle size, as well as different DS are also shown in **Figure 2.13**. The diffraction pattern of the CMC with DS 0.21, 0.38, 1.52, 2.21 and 2.41 are shown in **Figure 2.13a - e**, respectively. **Figure 2.13a** shows the diffractogram of CMC with crystallinity 59.33% and b shows CMC with crystallinity 56.66%, afterwards crystallinity decreases or almost disappears. The highest intensity peak is caused by the cellulose crystalline structure, while the amorphous portion causes the background noise line.

It can be seen from the **Figure 2.13** that crystallinity of CMC decreases with the increase of the DS from 0.21 to 2.41. According to Lin *et al.* [1990], higher DS of CMC results in decrease in crystallinity up to 1.1 values of DS, after that the crystallinity almost disappears. The same trend was observed with our samples.

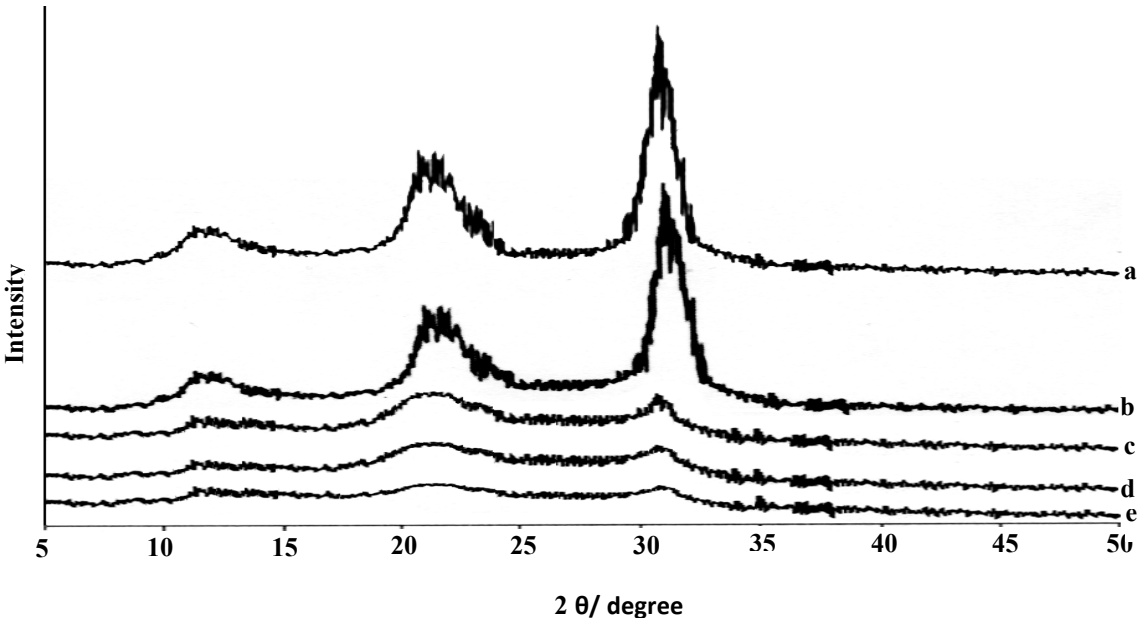


Figure 2.13 X-Ray diffractogram of synthesized CMC. (a) CMC with DS 0.21, (b) CMC with DS 0.38, (c) CMC with DS 1.52, (d) CMC with DS 2.21, (e) CMC with DS 2.41.

- **Thermal analysis**

Thermogravimetric analysis is a method used to measure the thermal stability of materials. The weight change with temperature was measured and used to infer the moments of change during the heating. The temperature at which the rate of maximum degradation occurs may be taken as an indicator of the stability of the material in comparative study. TGA curves of the extracted α-cellulose and synthesized CMC with DS 2.41 in nitrogen atmosphere are shown in **Figure**s **2.14** and **2.15**, respectively.

In **Figures 2.14** and **2.15**, the weight loss of moisture, volatile compounds and carbohydrate polymers during the carbonization phase are shown. The α-cellulose contains 4.4% moisture whereas the CMC contains 10.8% moisture, respectively. This shows that CMC is hygroscopic. The weight loss of α-cellulose is 72.5%, in the temperature range from 250°C to 370°C, depicted in **Figure 2.14**. The shoulder at about 326°C was due to the thermal decomposition of glycoside linkages of cellulose and the main peak at about 370°C to the decomposition of α-cellulose [Seo *et al.,* 2007; Aziz and Ansell, 2004].

On the other hand, the main decomposition of the synthesized CMC starts at above 250°C as demonstrated in **Figure 2.15**. The first stage was attributed to release of moisture, as hydrogen-bound water, to the CMC structure. The second and third stages of decomposition took place at 285°C and 318°C, respectively. Weight loss is 41.2%, probably owing to depolymerization, with formation of H_2O, CO, CO_2 and CH_4 [Durcilene *et al.,* 2007]. As there are COO^- groups in CMC, it is decarboxylated in this temperature range. The rate of weight loss increased with the increase in temperature.

In addition, DTG curves represent the thermal decomposition temperature, as in **Figures 2.14** and **2.15**. According to the vertex of **Figures 2.14** and **2.15**, the higher weight loss was found at 351.9°C and 302°C, respectively. The weight loss of α-cellulose was higher than that of synthesized CMC reported in **Figures 2.14** and **2.15**, respectively. These values are 80 μg / °C and 77.7 μg / °C.

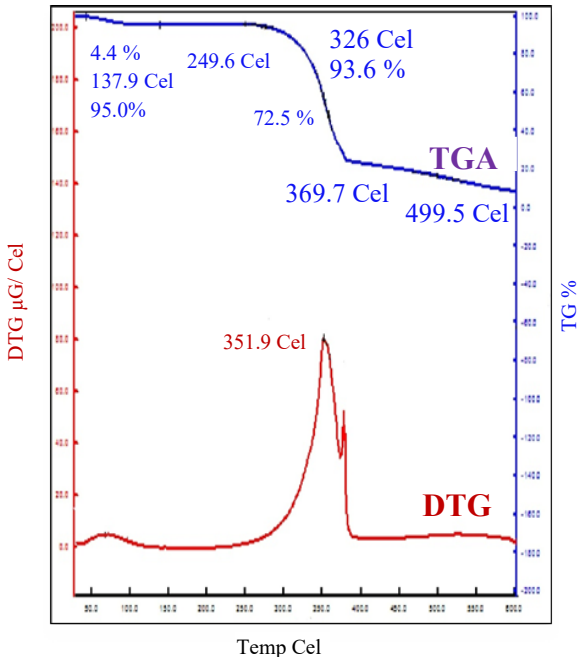


Figure 2.14 TGA and DTG curves of extracted α-cellulose.

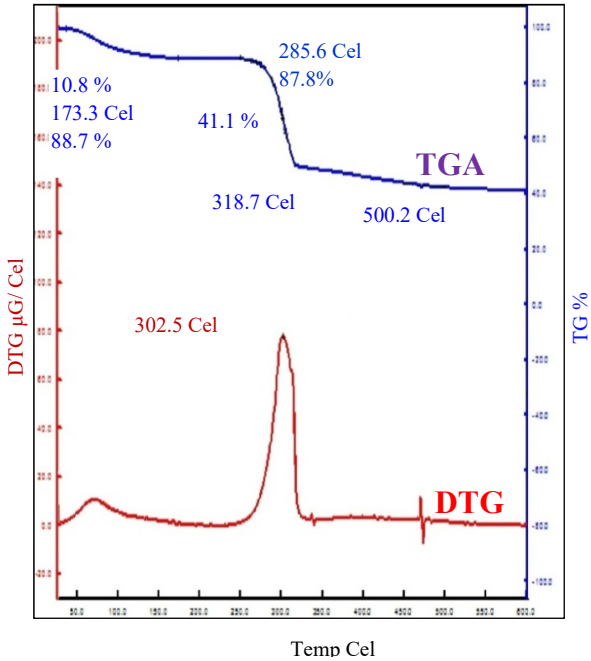


Figure 2.15 TGA and DTG curves of synthesized CMC with DS 2.41.

➢ Morphological analysis

The scanning electron micrograph (SEM) has several potential advantages in morphological investigation. This tool has been applied extensively in the fields of metallurgy, natural and modified fibers, polymers, biomaterials, composites [Sikdar *et al.,* 1995] etc. SEM analysis is a technique for examining granules morphology since it has a large depth of field, which allows a large amount of sample to focus at one time. The SEM also produces images of high resolution, which means that closely spaced features can be examined at high magnification.

The surface morphologies of extracted cellulose and synthesized CMC with DS 2.41 are shown in **Figures 2.16a and 2.16b**, respectively. **Figure 2.16b** illustrates that the obtained products are rod-like (or ribbon shaped), which is similar to other reported images for a typical CMC molecule [Ahemen *et al.,* 2013].

The surfaces of α-cellulose are smoother, with very minimal damage, whereas the prepared CMC surfaces are more extended, rough and collapsed [Rachtanapun *et al.,* 2012] as shown in **Figure 2.16a** and **2.16b**, respectively. This is because alkali-treated α-cellulose was further treated with alkali during carboxymethylation, resulting in the rupture of the surface of obtained CMC [Donald *et al.,* 2001]. Figures clearly show that the conversion of cellulose to CMC lead to change in its ribbon shape.

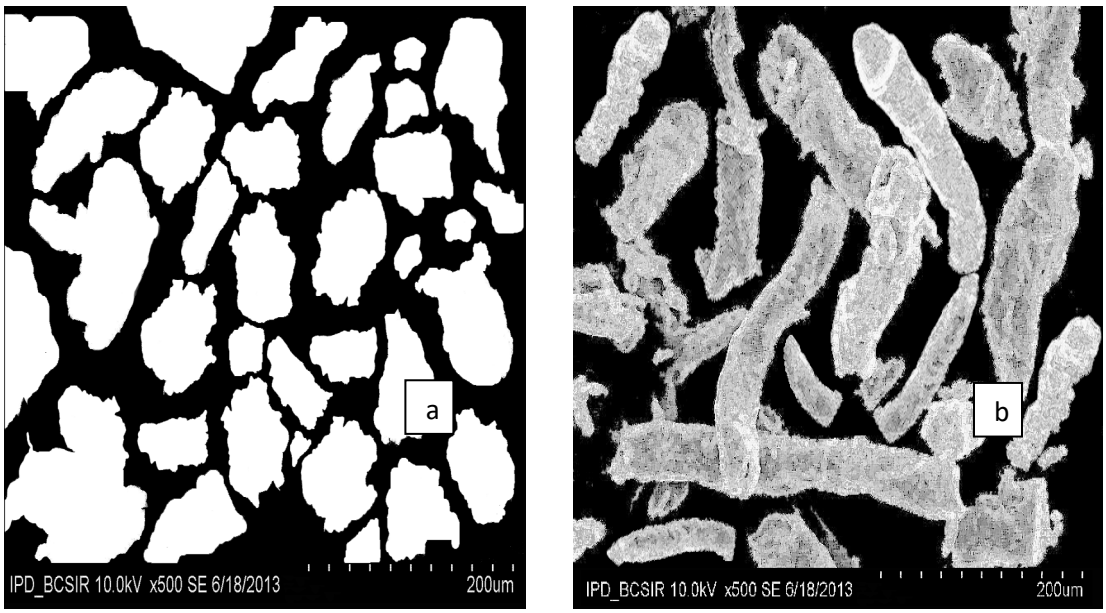
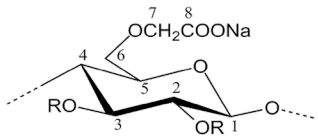


Figure 2.16 SEM image of (a) cellulose and (b) CMC at 500 magnification (200 µm size bar).

- **^{13}C NMR analysis**

This experiment gives a single sharp peak for each type of carbon in the molecule. The location of a particular peak is dependent on the chemical shift. Many factors affect chemical shift of a peak, mainly it can be explained by considering the electron density around the nuclei involved. The higher the electron density around

the nucleus, the further downfield (shifted to the left) the peak will appear in the spectrum. The ^{13}C NMR spectra of prepared CMC are shown in **Figure 2.17**.



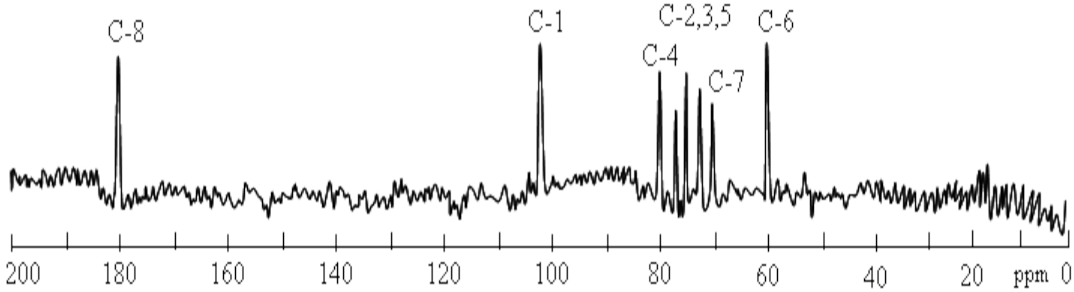


Figure 2.17 The ^{13}C NMR spectra of CMC film with a degree of substitution 2.41 recorded in D_2O at 25°C (number of scans 13223).

Figure 2.17 shows representative ^{13}C NMR spectra of CMC film with a DS value of 2.41. The spectra prove the molecular structure of CMC. Indeed, there are typical signals of the modified anhydroglucose unit (AGU) in the range of 60-105 ppm as well as peaks assigned to carboxymethyl groups from 178 to 180 ppm (C=O) as made by different groups of researchers [Baar *et al*., 1994; Ramos *et al*., 2005; Chadlia *et al*., 2006; Capitani *et al*., 2000; Chaudari *et al*., 1987]. From the Figure, it can be seen that the chemical shift of C-2, C-3, C-5 and C-6 occurred at 76.67 ppm, 74.31 ppm 72.62 and 60.24 ppm respectively. Also the chemical shift of C-8, C-1, C-4 and C-7 were at 179.28 ppm, 103.13 ppm, 80 ppm and 70.68 ppm respectively. Here the hydroxyl groups on C-3 and C-2 were only partially etherified and etherification mainly occurred on the hydroxyl groups on C-6.

2.4 CONCLUSION

While corn husks have been used on a small scale as a fuel for direct combustion in cooking and heating, their use as chemical feedstock for large-scale production is a more modern concept. Yet this concept has potential to help in reducing the pollution problem. α-cellulose was extracted from corn husks and etherification reaction was carried out by the infusion of cellulose with NaOH and monochloroacetic acid. High purity food-grade CMC was successfully produced as an additive for pharmaceutical and food industries.

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CHAPTER 3

TOXICOLOGICAL STUDY OF CMC

3.1 INTRODUCTION

Toxicology is the study of adverse affects of chemicals in people, animals and the environment. These chemicals include food additives, pesticides and food dyes in the food or medicines which people take every day. In addition to therapeutic agents, toxicologists examine many environmental agents and chemical compounds that are synthesized by humans or that originate in nature. The toxic effects of these agents may range from disturbances in growth patterns, discomfort, disease or death of individual organisms or on whole ecosystems.

Toxicology focuses more on the adverse effects of chemicals and risk assessment. Anything that is used by people or animals must be tested. More than 10,000 chemicals are allowed to be added, directly or indirectly, to human food as additive pursuant to the United States (US) Food Additives Amendment of 1958 as administered by the U.S. Food and Drug Administration [Neltner *et al.,* 2011].

Food additives are substances added to food to preserve flavor or enhance its taste and appearance, to thicken and stabilize various foods through formation of a gel. When the food is to be stored for a prolonged period, use of additives and preservatives are essential in order to maintain its quality and flavor.

The excess water in the foods can cause the growth of bacteria, fungi and yeasts. Use of additives and preservatives prevents spoiling of the foods due to the growth of bacteria and fungi. Additives and preservatives maintain the quality and

consistency of the foods. They also maintain palatability and wholesomeness of the food, improve or maintain its nutritional value, control appropriate pH, provide leavening and colour, and enhance its flavor.

There are many foods products that are made entirely from chemicals. As of 2010, over 90% of these additives were allowed in human food under the legal categories known as “food additives” or as “generally recognized as safe” (GRAS) substances. The remaining 10% are in smaller categories such as colour additives, pesticides, or substances whose use the federal government sanctioned before the law was enacted in 1958.

By law, food additives and GRAS substances (collectively referred as chemical additives) cannot be used in food without an affirmative determination that their use is safe (21 U.S.C. §321 and §348) by FDA or, in some cases, the additive manufacturer. Safety means that there is a “reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use” (21 CFR §170.30(i)) [Neltner *et al.,* 2013].

Toxicity testing requirements for assessing the safety of food; colour and food additives used in food have evolved over the past years as knowledge in the field of toxicology has expanded. While short-term or acute studies were considered adequate even for major food additives several decades ago, today's recommendations generally include comprehensive, long-term toxicity studies.

Toxicity evaluation is usually classified according to chronological scale that accounts for both dosage and response. Acute toxicity refers to effects of a single dose or multiple doses measured during a 24 h period. Toxic effects apparent over a period

of several days or weeks (14 or 28 days) are classified as subacute. Sub-chronic toxicity refers to toxic effects that occur between 30 days and 90 days exposure. Chronic effects are those produced by prolonged exposures of 3 months to a lifetime.

With the increasing use of processed foods since the 19th century, there has been a great increase in the use of food additives of varying levels of safety. Hamid *et al.*[2008] reported that to determine the safety of a chemical or drug for human use, toxicological evaluation is carried out in experimental animal to predict toxicity and to provide guidelines for selecting a ‘‘safe’’ dose in humans. The safety of regular CMC is well established [JECFA, 1990; SCF, 1992a].

The present study carried out to determine the biochemical, haematological and histopathological toxicity of synthesized CMC after sub-chronic oral administration in mice, with the aim to obtain information on the safety of CMC and provide guidance for selecting a safe dose in human use and exposure to this substance.

In recent years, the use of CMC as food additive in foods has extensively increased in Bangladesh as well as in the world. In order to evaluate its safety, the toxic effects of CMC on central nervous system development in mice were investigated. Furthermore, its 90-days repeated-dose sub-chronic toxic effect on mice of both sexes was studied to complete safety evaluations.

The purpose of toxicity study is two-fold, that is, to find out the effect caused and the level of exposure at which the effect is observed. Long-term toxicity studies are designed to detect effects on organs or body systems and the dose range over which the effect develops due to repeated administration for long period [Anonymous,

2005]. The data collected from toxicity studies will help to understand dose-toxicity response and to establish a safe dose for use in clinical trials.

Hence, present study was undertaken with an objective to evaluate the toxicological profile, identify the target organs of toxicity and to establish NOAEL after administration of CMC in mice for 90 consecutive days. This study was performed to establish the safety by conducting a repeated dose 90 day oral toxicity study in accordance to OECD Guideline No. 408 and WHO Guidelines [OECD, 1998; WHO, 2000].

In this chapter, an effort will be made to describe the general procedure of some methods those used in this experiment. The chapter begins with introduction or general principles of toxicology, which are important to the consideration of most topics in the field. The present chapter describes the detail results of the sub-chronic oral toxicity study.

3.2 MATERIALS AND METHODS

3.2.1 Materials

➢ ***List of chemicals***

No.	Chemicals	Producer
1.	Sodium chloride (NaCl, 99%)	Merck (Germany)
2.	Diethylether [$(C_2H_5)_2O$, 99%]	Merck (Germany)
3.	Nitric acid (HNO_3, 65%)	Merck (Germany)
4.	Ethanol (C_2H_5OH, 95%)	Merck (Germany)
5.	Sulfuric acid (H_2SO_4, 98%)	Merck (Germany)
6.	Perchloric acid ($HClO_4$, 72%)	Merck (Germany)
7.	Chloroform ($CHCl_3$, 99%)	Merck (Germany)
5.	Formalin (HCHO, 36%)	Merck (Germany)
6.	Xylene [$C_6H_4(CH_3)_2$, 98.5%]	Sigma (Switzerland)
7.	Picric acid [$(O_2N)_3C_6H_2OH$, 98%]	Sigma (Switzerland)
8.	Sodium hydroxide (NaOH, 98%)	Sigma (Switzerland)
9.	Phenol (C_6H_5OH, 99.5%)	Merck (Germany)
10.	Glucose ($C_6H_{12}O_6$, 99%)	Merck (Germany)
11.	Ethylene diamine tetraacetic acid [$(HOOCCH_2)_2NCH_2CH_2N(CH_2COOH)_2$, 99%]	Merck (Germany)
12.	Xylol [$C_6H_4(CH_3)_2$, 99%]	Merck (Germany)

- ***List of test kits***

No.	Test Kit	Producer
1.	Serum glucose detection kit	Randox, UK
2.	Cholesterol detection kit	Randox, UK
3.	Triglyceride detection kit	Randox, UK
4.	Glucose detection kit	Randox, UK
5.	Serum creatinine detection kit	Randox, UK
6.	Alkaline phosphatase detection kit	Biosystems, spain
7.	Serum urea detection kit	Human, Germany
8.	Glutamate pyruvate transaminase detection kit	Biosystems, spain
9.	Glutamate oxaloacetate transaminase detection kit	Randox, UK
10.	Spectrophotometric enzyme assay kits	Stanbio, USA
11.	Alanine aminotransferase detection kit	Stanbio, USA
12.	Aspartate aminotransferase detection kit	Stanbio, USA

- ***List of equipments***

1. Plastic cages with iron lids (for housing mice)
2. Water bottles (with drinking nozzles)
3. Blood collection tubes for serum
4. Eppendorf tubes
5. Scissors and forceps
6. Injection syringe with needle
7. Micropipettes and micropipette tips
8. Glass wire (volumetric flux, conical flux, beaker etc.)
9. Different plastic wares (pipette filler, micropipette tip holder, distilled water bottle)
10. Autoclave (ALP Co. Ltd. KT-30L, Tokyo, Japan)
11. -80°C freezer (Sanyo Electric Co. Ltd, Japan)
12. Centrifuge machine (eppendorf Model-5415)
13. Semi-automated bioanalyzer (Microlab-200 Vital scientific, Dieren, Netherlands)
14. Water bath
15. Incubator
16. Digital measuring balance
17. Measuring cylinder
18. Magnetic stirrer
19. Atomic Absorption Spectroscopy
20. Urine analyzer (Bayer 50, Germany).

3.2.2 Methods

This study was designed to meet or exceed the requirements of the following OECD (Organization for Economic Co-operation and Development) guidelines:

1. OECD: Guidelines for Testing of Chemicals (1981) Sub chronic Oral Toxicity –Rodent: 90-day Study, Guideline 408.
2. OECD: Guidelines for Testing of Chemicals (1997) Genetic Toxicology: Bacterial Reverse Mutation Test, Guideline 471.
3. OECD: Guidelines for Testing of Chemicals (1997) Genetic Toxicology: In vitro Mammalian Cell Gene Mutation Tests, Guideline 476.
4. OECD: Guidelines for Testing of Chemical (1997) Genetic Toxicology: Mammalian Erythrocyte Micronucleus Tests, Guideline 474.

This study was also designed to meet or exceed the requirements of the FDA (United States Food and Drug Administration) 1993 "Redbook II" Toxicological Principles for the Safety Assessment of Direct Food Additives and Colour Additives Used in Food.

This study was conducted at a contrast research organization in compliance with: OECD Principles of Good Laboratory Practices ENV/MC/CHEM (98) 17, EEC Good Laboratory Practice Standards (Part 58 of 21 CFR).

This study also complied with all appropriate parts of the Animal Welfare Act Regulations: 9 CFR Parts 1 and 2; 1989 and 9 CFR Parts 3 Animal Welfare Standards; 1991.

3.3 Parameters studied for sub-chronic oral toxicity

The sub-chronic oral toxicity studies of extracted purified compounds were performed on normal mice, both male and female for 90 consecutive days. The mice were kept under keen observations throughout the treatment period. The following parameters were studied during this test-

1. Gross general observation
2. Measurement of body weight
3. Measurement of organ weight
4. Food and water intake
5. Urinalysis
6. Haematological profiles
7. Biochemical parameters
8. Histopathology of liver, kidney, heart and lungs

3.4 Experimental animals

Healthy adult Swiss albino mice of both sexes were used to carry out the experiments. The animals were purchased from International Center for Diarrheal Diseases Research, Bangladesh (ICDDR'B). Each animal was examined to confirm suitability for study. Criteria for suitability included acceptable physical examination, body weight and ophthalmoscopic examination. At initiation of treatment, animals were approximately 6-7 weeks old. The individual initial body weights ranged from 26-28 g for male (mean 27 g) and 25-27 g (mean 26 g) for females. The weight variation of animals was minute quantity.

Each mouse was identified with a metal ear tag bearing its assigned animal number. The assigned animal number, plus the study number, comprised the unique

number for each animal. In addition, each cage was provided with a cage card which was colour-coded for dose level identification and contained study number and animal number information. All procedures were maintained by the method Bentivegna and Whitney [2002].

3.5 Animal maintenance

Animals were kept under standard animal husbandry conditions maintaining following parameters:

(i) Cage: Mice cages were made by polycarbonate with steel wire tops and wood-dust bedding which was changed once a week.

(ii) Temperature, light and humidity: A constant room temperature of 25°C ±3°C, humidity 50-60% and a controlled day length, 14 h light and 10 h dark with 10~20 air changes per hour were maintained in the laboratory.

The studies were conducted in accordance with good laboratory practices (GLP) [OECD, 1988] and the US Food and Drug Administration [FDA, 1982].

3.6 Housing conditions

The animals were housed in a cross-ventilated room and kept under standard environmental condition. They were randomly selected and housed in suspended polycarbonate cages in groups of 5 animals per cage with wood dust bedding. Temperature and relatives humidity were monitored and maintained within the specific range. The mice were identified by colour marking. They were allowed to acclimatization for 7 days to the laboratory conditions before the experiment.

All animals were examined during the acclimation period to conform suitably for study and those considered unsuitable were eliminated. The experiment was

performed in accordance with the guidelines established by the European Community for the Care Use of Laboratory Animals and were approved by Institutional Animal Ethical Committee [IAEC, 2009] and the Organization for Economic Cooperation and Development guidelines [OECD, 1998].

3.7 Mice food

Commercially available food ingredients, pellet diet were purchased from ICDDR'B, Dhaka; mixed homogeneously and this homogeneous mixture was then used as normal food for experimental mice (control and treated groups). Fresh diets were provided weekly and dietary concentrations were adjusted weekly based on body weight and feed consumption data from the preceding week. Prior to initiation of the study, analyses of homogeneity and stability of CMC in low and high concentration diets were conducted. Confirmation analysis of all four dietary levels were assayed weekly for the first, second and third months of the study.

Mice were fed with a standard diet with free access to water before and during the experiment and maintained as per National Institutes of Health guidelines [Guide for the Care and Use of Laboratory Animals, 2011]. Each batch of diet was assayed for bacterial and chemical contaminants as per US EPA Guidelines [1979]. The drinking water was similarly assayed at regular intervals. The results of these analyses provided no evidence of contamination that might have compromised the study.

Mice were given ideal food comprising the following ingredients per 100 g of mixture.

Table 3.1 Composition of mice food.

Composition	Amount (g)
Ata (flour)	40
Matar dal powder	25
Skimmed milk powder	28
Soyabean oil	05
Salt mixture	01
Vitamins mixture	01

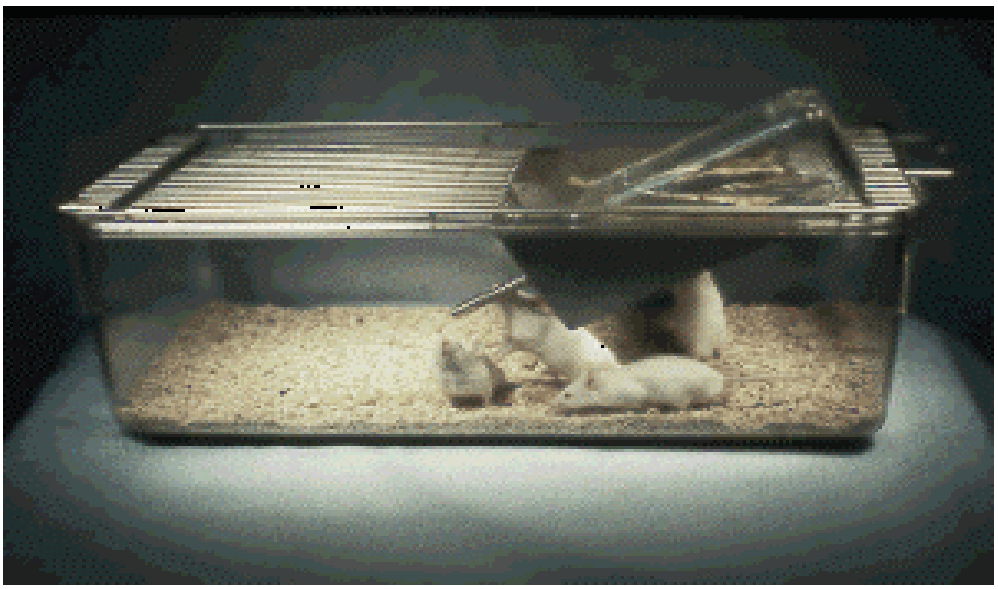
The diet supplied to each mice was about 4-5 g per day, which was approximately isocaloric. They were kept in a clean animal house with an optimal room temperature. The animals were maintained in this way for 15 days before administration of the compounds and continued upto the end of the experiment.

The nutrient composition of the diet is given in the **Table 3.2**.

Table 3.2 The nutrient composition of the diet (per hundred g of diet).

Nutrient	Amount (g)
Starch	66
Casein	20
Fat	8
Standard vitamins	2
Salt	4
	Total = 100

Mice food (pellet diet)



Mice in polycarbonate cage

3.8 Grouping of mice

Weight of the individual mice were determined and they were grouped into four groups such as A, B, C and D. Each group contains 10 mice comprised of 5 male and 5 female respectively. Group-A received diet with 2.5% CMC, group- B received diet with 5% CMC, and group-C received diet with 10% CMC and group-D received food with water only for control.

3.9 Preparation of dose

Test sample i.e. CMC powder administered orally with normal food to Swiss albino mice. The volume does not exceed 20 mg/g body weight per day. At least three doses of the test sample were taken. For group-A of mice, 125 mg of CMC powder was mixed with 5 g (per day for each mouse) of normal food. For group-B, 250 mg CMC powder was mixed and for group-C, 500 mg of CMC powder was mixed. The treatment schedule is as given below:

Group-A : 5 mg/g body weight/day

Group-B : 10 mg/g body weight/day

Group-C : 20 mg/g body weight/day

Group-D : Control

To adapt the animals of the high-dose groups to the experimental treatments, the doses of CMC were increased gradually from 2.5% on days 1-28 to 5% on days 28-60 and to 10% from days 60 onwards.

Table 3.3 Dosage regimen adjustments for each group of mice.

Group	No. of mice	Average body weight (g)	Sex	Average age (week)	Dose (i.p.) mg/mice/day
A	10	26	5 male and 5 female	6	125
B	10	27	5 male and 5 female	6	250
C	10	27	5 male and 5 female	6	500
D	10	26	5 male and 5 female	6	0 (control)

3.10 Sub-chronic oral toxicity study

Repeated dose 90-days oral toxicity studies were performed on Swiss albino mice with produced CMC as per the OECD guidelines no. 408 [OECD, 1998]. All animals were randomly assigned to the control and the three treatment groups. Animal care and handling conformed to accepted guidelines [OECD, 2002; OECD, 2008].

During the period of CMC administration, the animals were observed at least twice daily for signs of any toxicity, morbidity or mortality. Cage side behavioural observations, food and water consumption and urinalysis were conducted as a part of the regular checkup of the animals. Body weights, feed and water consumption of each animal were recorded at the start of study and thereafter at weekly intervals.

Evaluation parameters

3.11 Gross general observation

The mice were administered the compound with diet and were observed very keenly the following features:

1. Skin and fur
2. Eyes
3. Mucous membrane
4. Behaviour
5. Salivation
6. Lethargy
7. Sleep
8. Excitation
9. Depression
10. Diarrhea
11. Coma
12. Morbidity
13. Tremors/convulsions

Cage-side observations for morbidity and mortality were made at least twice daily (once in the morning and once in the afternoon). Cage side observations for general conditions were made once daily, concurrent with one of the viability checks, for any signs of toxic or pharmacologic effects (e.g., abnormalities in general condition, appearance, activity, behaviour, respiration). Animals were removed from their cages and examined behavioural and physical observations following the method described by DeMerlis *et al.* [2005].

3.12 Mortality and clinical signs

All animals were observed thoroughly for the onset of any immediate signs of toxicity and throughout the observation period to record any delayed acute effects and mortality. Clinical signs and mortality pattern were observed before dosing, immediately after dosing and up to 2-4 h after dosing during the thirteen-week dosing period [Al-Mamary *et al.,* 2002].

3.13 Measurement of body weight

The body weights of the animals were evaluated and recorded using a sensitive balance [OECD, 1995]. The mean body weight of each group was measured each day during the study. The mean body weight of mice was analyzed by ANOVA, and p values of <0.05 were considered to indicate a significant difference between groups [Hirbod Bahrani *et al.,* 2004].

3.14 Measurement of organ weight

Following euthanasia, organs (liver, kidneys, spleen, stomach, heart, adrenals, testes (male), uterus, cervix, and ovaries (female)) of mice in the various groups were excised on days 90, immediately separated, trimmed of any extraneous tissues, placed on a saline soaked gauze pad to retard desiccation and were immediately weighed (paired organs were weighed together) to one decimal place and calculated for organ weight ratio [Akhtar *et al.,* 2009].

$$\text{Organ weight ratio} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \times 100$$

3.15 Measurement of food consumption and water intake

The amount of food was measured before it was supplied to each cage, and food remaining was measured weekly thereafter (±2 days) to calculate the daily food consumption (g/mice/day). Feed was available without restriction 7 days. Animals were presented with full feeders of known weight. After 6 days, feeders were reweight and the resulting weight was subtracted from the full feeder weight to obtain the weight (g) consumed per animal over the 5 to 7 days period. Food consumption was measured (weighed) weekly, beginning one week prior to treatment. Average weight (g) of food consumed per kg of body weight per day (g/kg body weight/day) was calculated for each feeding period by first dividing the weight (g) of food consumed by the number of days (5 - 7) in the feeding period. The resultant value for average feed consumed per day was then divided by the average of the current and previous body weight of the animal.

The amount of water consumed were measured daily from the quantity of water supplied and the amount remaining after 24 h. All experiments were done using method following by Bentivegna and Whitney [2002].

3.16 Measurement of urinary parameters

Urine was collected for routine analysis at various times from each group of male and female mice on days 30, 60 and 90. For urine collection, mice were placed overnight in individual metabolism cages and urine was collected over a period of 16 h. The animals had access to water but not to food. The collected urine was kept in dry-ice cooled beakers. After collection, the urines were thawed, vigorously mixed and analyzed for different urinary parameters [Bar *et al.,* 1995]. Chemical analyses were performed using reagent strips `Combur-9-test RL' and an Urotron RL 9

reflection photometer. Specific gravity was measured with a refractometer and urine sediments were examined microscopically [Klimisch *et al.,* 1997].

The following parameters were studied:

1. Urine volume
2. Density
3. pH
4. Sodium (Na)
5. Potassium (K)
6. Chloride (Cl^-)
7. Calcium (Ca)
8. Phosphorus (p)

3.17 Collection of blood and serum samples

Blood samples were collected from the tail veins of each group of mice by conventional way on days 30, 60 and 90. It was then kept at room temperature for 30 min to clot. Afterwards, the test tube containing the clotted blood sample was centrifuged at 3000 r/min for 10 min using a table centrifuge to enable a complete separation of the serum from the clotted blood. The clear serum supernatant was then carefully aspirated with syringe and needle and stored in a clean sample bottle for the biochemical tests [Kumar and Sastry, 2010]

3.18 Monitoring of haematological profiles

The haematological profiles of the control and experimental mice were done to check the haematological abnormalities after the administration of the purified compounds orally. For this purpose, the following parameters were observed:

i. Total R.B.C. count

ii. Total W.B.C. count

iii. Differential count of W.B.C.

iv. Platelet count

v. Hemoglobin percentage

vi. Lymphocytes

vii. Neutrophils

viii. Monocyte

Procedure:

(i) Blood was collected into heparinised bottles and allowed to clot.

(ii) Collected blood was taken into tubes containing ethylene diamine tetraacetic acid (EDTA) anticoagulant.

(iii) Blood smears were made on glass slides and stained with leishmen reagent to perform TC, DC and platelet count. With the use of capillary tubes, blood was drawn to estimate the hemoglobin percentage by the Cyameth-haemoglobin method described by Grossland [1980]. The white blood cells (WBC) and red blood cells (RBC) were estimated using the improved Neubauer counting chambers as described by Dacie and Lewis [1991]. Neutrophil, lymphocyte and monocyte were determined as described by Dacie and Lewis [1995].

(iv) Post haematological studies were done on 30, 60 and 90 days after the commencement of drug administration following the same procedure as that done on normal rats.

All haematological parameters were determined at room temperature, (27±0.5) °C.

3.19 Estimating the biochemical parameters of blood

The biochemical parameters such as SGOT (Serum-glutamate-oxaloacetate-transaminase), SGPT (Serum-glutamate-pyruvate-transaminase), SALP (Serum alkaline phosphatase) and serum bilirubin are associated with the condition of liver, while serum level of creatinine and urea are associated with the functioning of kidney. Serum levels of these parameters change with the pathological changes of these organs. In the case of hepatic necrosis, cirrhosis and obstructive jaundice, the serum level of SGOT and SGPT may increase upto 200 IU/L. If a compound possesses any effect on kidney, several pathological changes may occur and ultimately serum level of these parameters altered.

The following parameters were checked

I. Liver function tests

i. SGOT

ii. SGPT

iii. ALP

iv. Serum bilirubin

II. Kidney function tests

i. Serum creatinine

ii. Serum urea

III. Other biomolecules

i. Serum cholesterol

ii. Serum triglycerides

iii. Serum glucose

Serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also estimated using spectrophotometric enzyme assay kits (Stanbio, USA). The test was done using the procedure and reagents described in Boehrienger Mannheim GmbH Diagnostic.

Procedure

A. Collection of serum

On the targeted day of treatment with the compounds, the mice of experimental and control groups were sacrificed by a surgical blade No. 22 and the blood was collected in plastic centrifuge tubes. These were then allowed to clot at 40°C for 4 h. After clotting, the blood samples were centrifuged at 4000 rpm for 15 min using a WIFUNG centrifuge LABOR-50M, Germany**.** The clear straw colour serum was then collected in vials with Pasteur pipette and stored at 20°C.

B. Analysis of the serum for enzyme content

The serum thus obtained was used in the determination of biochemical parameters were measured using Randox Laboratories, UK reagent kits.

- ***Estimation of serum glucose***

Serum glucose was estimated by Glucose-Oxidase (GOD-PAP) method by the Automatic Analyzer (Hitachi Ltd., Tokyo, Japan) using reagents of Randox Laboratories Ltd., UK. The principle of serum glucose measurement is described below:

Glucose was determined after enzymatic oxidation in the presence of glucose-oxidase (GOD). The hydrogen peroxide formed reacts under the catalysis of peroxidase (POD), with phenol and 4-aminophenazone to form a red violet

quinoneimine dye as indicator. The intensity of the colour formed is proportional to glucose concentration in the sample [Barham and Trinder, 1972].

Reagents

Reagent	Composition
Buffer	Phosphate buffer+
	Phenol
GOD-PAP Reagent	4-aminophenezone
	Glucose oxidase
	Peroxidase
Standard	Glucose

Reactions

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-amino phenazone} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quioneneimine} + 4\,H_2O$$

Calculation

Calculation of result for unknown sample is as follows:

Concentration of glucose in the sample

= (Absorbance of sample/Absorbance of standard) x (Standard concentration)

= mg/dL cholesterol in the sample

Conversion factor: mg/dL x 0.0258 = mmol/L

All blood samples were analyzed in duplicate and then mean values were taken.

➢ ***Estimation of serum cholesterol***

Serum total cholesterol was measured by enzymatic endpoint method (Cholesterol Oxidase/Peroxides) in the Automatic Analyzer (Hitachi 704, Hitachi Ltd., Tokyo, Japan) using reagents of Randox Laboratories Ltd., UK. Principle of the method is briefly described below:

Cholesterol esterase (CHE) hydrolyses the cholesterol esters present in the serum specimen to form cholesterol and fatty acids. Free cholesterol was then oxidized by cholesterol oxidase (CHOD) to 4-Cholestenon and hydrogen peroxide (H_2O_2). Hydrogen peroxide reacts with 4-aminophenazone (4-AP) and phenol in the presence of peroxidase (POD) to produce a coloured substance, quinoneimine. The intensity of the colour formed is proportional to cholesterol concentration in the sample which has been measured through analyzer at 500-550 nm [Trinder, 1969].

Reactions

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{4-Cholestenon} + H_2O_2$$

$$2H_2O_2 + \text{4-AP} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

Reagents

Reagent	Composition
Buffer	Pipes pH 6.9
	Phenol
Enzymes	Peroxidase
	Cholesterol esterase
	Cholesterol Oxidase
Standard	Cholesterol aqueous

Calculation

Calculation of result for unknown sample is as follows:

Concentration of cholesterol in the sample

= (Absorbance of sample/Absorbance of standard) x (Standard concentration)

= mg/dL cholesterol in the sample

Conversion factor: mg/dL x 0.0258 = mmol/L

All blood samples were analyzed in duplicate and then mean values were taken.

- ***Estimation of serum triglyceride***

Serum triglyceride was measured by enzymatic colourimetric method. The principal of this method is as follows:

The triglyceride was determined after enzymatic hydrolysis of triglycerides by lipases to form glycerol and free fatty acids. The glycerol formed in reaction 1 was phosphorylated to glycerol-3-phosphate in a reaction catalyzed by glycerol kinase (GK) (eq 2). The glycerol-3-phosphate was oxidized by glycerol phosphate oxidase (GPO) producing dihydroxyacetone phosphate and hydrogen peroxide (eq 3). A brilliant purple coloured (eq 4) substance, quinoneimine dye was formed from hydrogen peroxide (H_2O_2), 4-aminoantipyrine (4-AAP) and N-Ethyl-N-(3-sulfopropyl)-m-anisidine (ESPA) under the catalytic influence of peroxidase. The intensity of the colour formed is proportional to triglyceride concentration in the sample. The absorbance is measured at 540 nm [Bucolo and David, 1973; Fossati and Prencipe, 1982; McGowan, 1983].

Reactions

Triglycerides $\xrightarrow{\text{Lipase}}$ Glycerol + fatty acid…….....……..…(1)

Glycerol + ATP $\xrightarrow{\text{GK}}$ Glycerol-3-phosphate + O_2 + ADP….....…(2)

Glycerol-3-phosphate+ O_2 $\xrightarrow{\text{GPO}}$ Dihydroxyacetone phosphate + H_2O_2…..(3)

$2H_2O_2$+4-Aminoantipyrine +ESPA $\xrightarrow{\text{Peroxidase}}$ Quinoneimine + $4H_2O$…… (4)

Reagents

Reagent	Composition
Buffers	Tris
	Borate
	Phosphate
Enzymes	EDTA
	Trypsin
	Antipain
Standard	Triglyceride glycerol

Calculation

Calculation of result for unknown sample is as follows:

Concentration of triglyceride in the sample

= (Absorbance of sample/Absorbance of standard) x (Standard concentration)

= mg/dL triglyceride in the sample

Conversion factor: mg/dL x 0.0258 = mmol/L

All blood samples were analyzed in duplicate and then mean values were taken.

➢ ***Estimation of serum urea***

Urea is hydrolyzing in the presence of water and urease enzyme to produce NH_4^+ and CO_2. Produced NH_4^+ in the first reaction combines with α-ketoglutarate and NADH in the presence of glutamate dehydrogenase (GLDH) to yield glutamate and NAD^+ [Teitz, 1995; Fawcett and Scott, 1960; Mackay and Mackay, 1972].

Reactions

$$\text{Urea}+H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + CO_2$$

$$2NH_4^+ + 2\text{-}\alpha\text{-ketoglutarate} + 2NADH \xrightarrow{\text{GLDH}} 2\text{L-Glutamate} + 2NAD^+ + 2H_2O$$

Reagents

Reagent	Composition
R_1:	Urease GLDH NADH
R_2:	Tris buffer (pH 8.0) α-ketoglutarate

Calculation

Concentration of urea in the sample

= (Absorbance of sample/Absorbance of standard) x (Standard concentration)

= mg/dL urea in the sample

Conversion factor: mg/dL x 0.0258 = mmol/L

All blood samples were analyzed in duplicate and then mean values were taken

- ***Estimation of serum creatinine***

Creatinine forms an orange coloured complex with picric acid in alkaline solution. The intensity of the obtained colour is directly proportional to the creatinine concentration in the sample [Weil, 1952].

Reaction

Creatinine + Picric acid (Yellow) $\xrightarrow{\text{NaOH}}$ Creatinine-picrate complex (Orange)

Reagents

Reagent	Composition
R_1:	Picric acid
R_2:	Sodium hydroxide
Standard	Creatinine

Calculation

To calculate the activity of serum creatinine, the following formula was used according to the protocol:

Creatinine (mg/dL)= $(\Delta Abs_{sample}/\Delta Abs_{standard}) \times 2.0$

Where,

Δ = changes of absorbance per minute at 405 nm.

All blood samples were analyzed in duplicate and then mean values were taken.

- ***Estimation of alkaline phosphatase***

Serum alkaline phosphatase (ALP) estimation procedure is based on the method developed by Bowers and McComb(1975) and has been formulated as recommended by the AACC and IFCC (Tietz, 1986).

Alkaline phosphatase (ALP) enzyme hydrolyses p-nitrophenylphosphate (pNPP) into inorganic phosphate and yellow coloured p-nitrophenol in the presence of 2-amino-2-methyl-1-propanol (AMP) at pH 10.4. The rate of change in absorbance due to the formation of pNP was measured bichromatically at 480 nm. The intensity of the colour developed is directly proportional to the ALP activity in the sample.

Reaction

$$\text{pNPP} + H_2O + \text{AMP} \xrightarrow{\text{ALP}} \text{p-nitrophenol (pNP)} + \text{AMP-}PO_4$$

The unit of enzyme activity is defined as the micro molecules of pNPP hydrolyzed per min per ml of serum.

Reagents

Reagent	Composition
R_1:	P-nitrophenyl phosphate
R_2:	AMP buffer (pH 10.5) $MgCl_2$

Calculation

To calculate the activity of serum ALP, the following formula was used according to the protocol:

$$\text{ALP (IU/L)} = (\Delta Abs_{sample}/min - \Delta Abs_{blank}/min) \times F$$

Where,

$F = V_t/(V_s \times \text{extinction coefficeint}) \times 1000$

ΔAbs/min = Average absorbance change per minute

1000 = Conversion of IU/ml to IU/L

V_t= total reaction volume (ml)

V_s= sample volume (ml)

All blood samples were analyzed in duplicate and then mean values were taken.

- ***Estimation of serum glutamate oxaloacetate transaminase***

In this process, L-Aspartate and α--Ketoglutarate react in the presence of AST (aspartate transaminase) in the sample to yield oxaloacetate and L-glutamate. The Oxaloacetate is reduced by malate dehydrogenase (MDH) to yield L-malate with the oxidation of NADH to NAD. The reaction is monitored by measurement of the decrease in absorbance of NADH at 340 nm. The rate of reduction in absorbance is proportional to serum glutamate oxaloacetate transaminase (SGOT) activity in sample [Tietz, 1986; Bergmeyer *et al.,* 1986; Wallnofer *et al.,* 1974; Thefeld *et al.,* 1974].

$$\text{L-Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-glutamate.}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD} + H_2O$$

Reagents

Reagent	Composition
R1:	α-Ketoglutaric acid Malic dehydrogenase (MDH) Lactic dehydrogenase (LDH)
R2:	Tris-Buffer (pH 7.8) L-aspartic acid

Calculation

To calculate the activity of serum SGOT, the following formula was used according to the protocol:

SGOT (IU/L)= $(\Delta Abs_{sample}/min - \Delta Abs_{blank}/min) \times F$

Where,

$F=V_t/(V_s \times \text{extinction coefficeint}) \times 1000$

$\Delta Abs/min$ = Average absorbance change per minute

1000 = Conversion of IU/ml to IU/L

V_t=total reaction volume (ml)

V_s= sample volume (ml)

All blood samples were analyzed in duplicate and then mean values were taken.

- ***Estimation of serum glutamate pyruvate transaminase***

In this process, L-alanine and alpha-ketoglutarate react in the presence of alanine aminotransferase (ALAT) in the sample to yield pyruvate and L-glutamate. Pyruvate is reduced by lactate dehydrogenase (LDH) to yield lactate with the oxidation of NADH to NAD^+. The reaction is monitored by measurement of the decrease in absorbance of NADH at 340 nm. The rate of reduction in absorbance is proportional to serum glutamate pyruvate transaminase (SGPT) activity in sample **[**Tietz, 1986; Bergmeyer *et al.,*1986**].**

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$$\text{L-alanine} + \alpha\text{-Ketogluterate} \xrightarrow{\text{ALAT}} \text{Pyruvate} + \text{L-Glutamate.}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+ + H_2O.$$

Reagents

Reagent	Composition
R1:	α-ketoglutaric acid (NADH) Lactic dehydrogenase (LDH)
R2:	Tris-buffer (pH 7.3) L-Alanine

Calculation

To calculate the activity of serum SGPT, the following formula was used according to the protocol:

SGOT (IU/L)=$(\Delta A_{sample}/min-\Delta A_{blank}/min)\times F$

Where,

$F=V_t/(V_s\times extinction\ coefficeint)\times 1000$

ΔAbs/min = Average absorbance change per minute

1000 = Conversion of IU/ml to IU/L

V_t=total reaction volume (ml)

V_s= sample volume (ml)

All blood samples were analyzed in duplicate and then mean values were taken.

3.20 Histopathology of liver, kidney, heart and lung

Histopathology of liver, kidney, heart and lung were performed to observe any changes in the cellular structures (degradation and regeneration) of the mice after receiving purified compounds at a dose of 125 mg/mice/day, 250 mg/mice/day and 500 mg/mice/day for 90 consecutive days with respect to control group. For

histopathological examinations, standard procedures were followed [Islam & Hoque, 2015].

Reagents

i. Formaline (10%)
ii. Absolute alcohol (ethanol)
iii. Paraffin
iv. Xylene
v. D.P.X. mounting fluid
vi. Harris hematoxylin and eosin stain

Procedures

A. Collection and processing of the tissues

The liver, kidney, heart and lungs were collected from different groups of treated and control mice. After end of the exposure period of 90 days, mice in each group were sacrificed by placing them in anaesthetic jar containing cotton wools soaked in chloroform. Complete anaesthesia was considered accomplished when the pedal movements and eyelid reflex disappeared and the animals became recumbent while still breathing. Detailed post mortem examinations were conducted by opening up of belly of the mice; gross lesions and internal haemorrhages were noted, the tissues were sliced into pieces, each measuring a few mm of thickness.

The sliced tissues were then immersed in 10% formalin for three days. The preserved tissue samples were washed in running tap water for a couple of h, dehydrated in ascending grades of ethanol (30%-95%), cleared in xylol and embedded in paraffin wax (melting point 50-56° C). After solidification the wax blocks were cut at 5µm thickness using a rotary microtome at 200 µm intervals and the small pieces of the ribbon were affixed on the slides. Finally, the slides with the sections were placed

in descending grades of ethanol (95%-70%), rinsed in distilled water for 2-3 min and then double stained in haematoxylin (10-15 min) and aqueous eosin (5-10 min), followed by further rinsing in distilled water.

B. Staining

The sections were deparaffinized by two changes of xylene (5 min /each) and hydrated in alcohol (2-3 min/ each) and then cleaned in two sets of xylene (5 min/ each).

C. Mounting

Glass slides containing the tissues were wiped, dried and then a drop of Canada balsam was put on the section and cover slip was gently placed on it. On the sections, thin film between the cover slip and the slide with the mounting medium (Canada balsam) was formed to attach them.

D. Microphotograph

Histopathological examination was done under high magnifying power Microscope (Zeiss, Germany) and microphotographs were taken at the Department of Biochemistry, University of Rajshahi, Bangladesh, using an automated digital camera system (Olympus CH30/CH40, Japan).

3.21 Statistical Analysis

Results were expressed as mean ± standard error of mean. The toxicological data were analyzed by ANOVA, followed by Dunnett multiple comparison test and two-sample *t*-test using the Minitab statistical software respectively. P values less than 5% were considered statistically significant ($p < 0.05$). Body weight data were analyzed using a one-way analysis of covariance, followed by Dunnett's multiple comparison tests [Dunnett, 1955, 1964; Cochran, 1957; Steel and Torrie, 1960].

One-way analysis of variance (ANOVA) was used in analyzing body weight change, organ weight, food and water consumption and quantitative urinary parameters. When group means were significantly different ($P < 0.05$), individual pair-wise comparisons were made using Dunnett's multiple comparison method [Dunnett, 1955, 1964; Siegel, 1956; Cochran, 1957; Steel and Torrie, 1960]. Independent of the ANOVA results, the homogeneity of variances was tested using Bartlett's test, and if significantly different ($P < 0.01$), Kruskal–Wallis non-parametric ANOVA was performed. Fisher's exact test was performed on all histopathology data [Siegel, 1956].

The instruments used during the studies are given below:

Laminar air flow

Autoclave

Incubator

Urine Analyzer

Centrifuge Machine

Semi- automated Bioanalyzer

3.22 RESULTS AND DISCUSSION

- ***General sign and behavioural analysis***

Evaluation of toxic properties of a substance is crucial when considering for public health protection because exposure to chemicals can be hazardous and results to adverse effects on human being [Asante-Duah, 2002]. The appearance and behavioural parameters of animals after drug or chemical administration is indicator of the toxicity of the test compounds [Kumar and Lalitha, 2013; Jothy *et al.,* 2011].

The toxic effect of prepared CMC on the appearance and the general behavioural pattern of mice are shown in **Table 3.4** and **Table 3.5.** No toxic symptoms or mortality were observed in any animals, which lived upto 90 days after the administration diet with CMC from dose level of 5 mg/g body weight to 20 mg/g body weight per day given in **Table 3.4.** The behavioural patterns of animals were observed first 6 h and followed by 14 h after the administration. The animals in both control and treated groups were normal and did not display significant changes in behaviour, skin effects, breathing, sleep pattern, impairment in food intake and water consumption, postural abnormalities and hair loss.

CMC is water soluble, yet only poorly fermented by the intestinal microflora [Bar *et al.,* 1995]. Because of their water binding and bulking effect, the product produces softer stools [Wyatt *et al.,* 1988]. During the study, animals with high dose-CMC produced softer stools. Similar laxative effect has been observed in other feeding studies with various non-digestible plant gums, low digestible carbohydrates or polyols [Bar *et al.,* 1995]. No significant changes were observed in wellness parameters used for evaluation of toxicity. In the treated group at the highest dose level of CMC (group-C) in first 6 h, rapid heartbeat was observed after the

administration, but it then become normal and this may due to the stress of receiving the oral administration of the product. There was no noticeable deviation in the behaviour of the mice treated with CMC doses of 5, 10 and 20 mg/g/day compared to that of the control. Both the control and treated mice appeared uniformly healthy at the end and throughout the 13 weeks period of study.

The clinical symptom is one of the major important observations to indicate the toxicity effects on organs in the treated groups [Eaton and Klaassen, 1996]. In this study, there was no morbidity or death observed at the highest dose of 20 mg/g body weight. The sub chronic toxicity study in mice showed that at 20 mg/g dose, the product is safe for consumption and for medicinal uses. In toxicity rating by joint FAO/WHO Expert Committee on Food Additives [WHO, 1966], if at 2 g/kg oral dose no death occurred, it is sufficient to assume the substance to be non toxic.

Table 3.4 Potential toxic effects of CMC in mice.

Control[A]	**Treated[B]**	**Treated[C]**	**Treated[D]**
0/10 [c]	0/10	0/10	0/10

D = control group (treatment without CMC); A,B,C = test groups (treatment with 5 mg/g, 10 mg/g and 20 mg/g CMC respectively); c = Number of dead mice/ number of mice used.

Table 3.5 General appearance and behavioural observations for control and treated groups.

Observation	Control group		Test group	
	6 h	**14 h**	**6 h**	**14 h**
Skin and fur	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal
Behavioural patterns	Normal	Normal	Rapid heart beat	Normal
Salivation	Absent	Absent	Absent	Absent
Lethargy	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal
Diarrhoea	Absent	Absent	Absent	Absent
Coma*	N.O.	N.O.	N.O.	N.O.
Death	No	No	No	No
Tremors	Absent	Absent	Absent	Absent
Convulsions	Absent	Absent	Absent	Absent
Other symptoms	Nil	Nil	Nil	Nil

*N.O. = Not Observed.

- ***Changes in body weight***

Changes in the body weight have been used as an indicator of adverse effects of drugs and chemicals [Nandy and Datta, 2012]. Generally, the alterations of body weight gain of mice would reflect the toxicity after exposure to the toxic substances [Carol, 1995] and it will be significant if the body weight loss occurred is more than 10% from the initial weight [Raja *et al.,* 2002; Teo *et al.,* 2002].

Table 3.6A and **3.6B** showed the change of body weight observed weekly after the administration of the test compound for male and female mice respectively. In this study, the body weight gain of control group was 27-31 mg for male mice and 25-29 mg for female mice; the test compound even at the highest dose level (20 mg/g/day) studied showed normal weight gain which was 28-31 mg for male mice and 25-29 mg for female mice. All animals exhibited a normal increment in body weight without drastic difference between both control and treated groups. Although, the body weights of all the mice were increased but, the changes of the body weights were found to be statistically insignificant. Insignificant increase in body weight of test animals indicates that the administration of the CMC had no toxic effect on animals. According to reports, reduction in body and internal organ weights are considered sensitive indices of toxicity after exposure to toxic substance (Raja *et al.,* 2002, Teo *et al.,* 2002; Thanaborn *et al.,* 2006).

Body weight is an important parameter indicating the animal's condition. It is the basis to determine the dose of anesthetics or test substances in toxicity studies [Song *et al.,* 1990]. However, this parameter is frequently affected by feeding, measurement time, and measurement sensitivity. In our study, the standard deviations of body weight at each age were less than 10%; thus, individual differences were small.

Table 3.6A Weekly body weight (g) record of male mice.

Duration in Week	Dose (mg/g body weight/ day)			
	0 mg/g/day (control group)	**5 mg/g/day**	**10 mg/g/day**	**20 mg/g/day**
0	27.80±2.80	27.12±2.00	28.11±2.80	28.18±1.90
1	27.78± 2.00	28.60± 2.01	27.37± 2.02	28.26± 2.00
2	28.10± 2.00	27.40± 2.10	29.39± 2.11	28.89± 2.18
3	27.98± 2.07	28.48± 2.17	28.94± 2.10	28.99± 2.17
4	27.92± 2.36	29.80± 2.46	28.00± 2.06	29.99± 2.44
5	27.78± 2.89	29.00± 2.89	27.95± 2.91	29.09± 2.89
6	28.03± 2.01	28.80± 2.00	27.91± 2.11	29.44± 2.01
7	29.30± 2.22	29.73± 2.31	28.83± 2.01	28.39± 2.40
8	29.94± 2.09	30.04± 2.11	28.94± 2.05	28.44± 2.09
9	30.46± 2.20	30.46± 2.11	30.40± 2.21	30.96± 2.22
10	29.97± 2.00	30.90± 2.08	29.99± 2.10	29.97± 2.01
11	30.03± 2.31	30.53± 2.33	30.93± 2.01	30.03± 2.31
12	30.03±2.10	30.83±2.12	31.03±2.11	30.09±2.00
13	31.76± 2.70	31.09± 2.61	30.87± 2.90	31.03± 2.81

Data presented as mean ± standard deviation (n = 5), at 5% level of significant ($p<0.05$). Statistical tests: Analysis of covariance followed by multiple comparison tests (Dunnett).

Table 3.6B Weekly body weight (g) record of female mice.

Duration in Week	Dose (mg/g body weight/ day)			
	0 mg/g/day (control group)	**5 mg/g/day**	**10 mg/g/day**	**20 mg/g/day**
0	25.10±2.80	25.12±2.00	25.11±2.80	25.18±1.90
1	25.50± 2.00	25.60± 2.01	25.37± 2.02	25.26± 2.00
2	25.10± 2.00	26.40± 2.10	25.39± 2.11	26.89± 2.18
3	26.48± 2.07	26.48± 2.17	25.94± 2.10	26.99± 2.17
4	27.92± 2.36	26.80± 2.46	26.00± 2.06	27.99± 2.44
5	27.78± 2.89	26.00± 2.89	26.95± 2.91	27.09± 2.89
6	27.0± 2.01	27.80± 2.00	26.91± 2.11	27.44± 2.01
7	26.3± 2.22	28.73± 2.31	27.33± 2.01	28.39± 2.40
8	28.94± 2.09	27.94± 2.11	26.94± 2.05	28.44± 2.09
9	28.46± 2.20	28.46± 2.11	27.40± 2.21	28.96± 2.22
10	28.97± 2.00	28.90± 2.08	27.59± 2.10	29.07± 2.01
11	28.03± 2.31	28.53± 2.33	27.63± 2.01	28.33± 2.31
12	28.03±2.10	29.03±2.12	28.93±2.11	28.39±2.00
13	29.76± 2.70	29.99± 2.61	28.87± 2.90	29.03± 2.81

Data presented as mean ± standard deviation (n = 5), at 5% level of significant ($p<0.05$). Statistical tests: Analysis of covariance followed by multiple comparison tests (Dunnett).

- ***Changes in organ weight***

Organ weight is also an important index of physiological and pathological status in animals. The relative organ weight is fundamental to establish whether the organ was exposed to the injury or not. The heart, liver, kidney, spleen, and lungs are the primary organs affected by metabolic reaction caused by toxicant [Akanmu *et al.,* 2004; Raphael and Obioma, 2014]. The liver, being a key organ in the metabolism and detoxification of xenobiotics, is vulnerable to damage induced by a huge variety of chemicals [Jothy *et al.,* 2001].

The weights of vital organs of the animals of both male and female were calculated and are recorded in **Table 3.7A** and **Table 3.7B** respectively. Table shows the effect of product on principal organ weights relative to body weight. There were no significant differences in the changes of each weight with increasing dose level of the test compound, yet organ weights were slightly higher in females of control, low-, mid-, and high-dose CMC groups. These significant increases were considered to be due to differences in gender. The results revealed that, the essential organs such as kidney, liver, heart, lung and spleen were not adversely affected throughout the treatment.

The weights of kidney were 0.46 g (male mice) and 0.55 g (female mice) for control groups; whereas 0.45-0.50 g (male mice) and 0.53-0.50 g (female mice) for treated groups. The weights of liver were 1.58 g (male mice) and 1.80 g (female mice) for control groups; whereas 1.60-1.61 g (male mice) and 1.84-1.81 g (female mice) for treated groups. The weights of heart were 0.20 g (male mice) and 0.25 g (female mice) for control groups; whereas 0.19-0.20 g (male mice) and 0.26-0.26 g (female mice) for treated groups. The weights of lung were 0.79 g (male mice) and 1.01 g

(female mice) for control groups; whereas 0.80-0.79 g (male mice) and 1.08-1.00 g (female mice) for treated groups. The weights of spleen were 0.09 g (male mice) and 0.10 g (female mice) for control groups; whereas 0.09-0.09 g (male mice) and 0.09-0.09 g (female mice) for treated groups. The weights of testes for both control and treated groups were 0.19 g and 0.19-0.20 g respectively. The weights of ovaries for both control and treated groups were almost similar, 0.05 g and uterus were 0.14-0.17 g (control-highest dose level).

The relative organ weight of mice between treated and control groups shown statistically significant differences ($P < 0.05$). This indicates the compound has not caused any adverse effect on normal physiological functions of the body. The similar findings were also reported by Jothy *et al*. [2001].

Table 3.7A Organ weight record of male mice (g) at terminal sacrifice (organ to body weight index%).

Organ	Dose (mg/g body weight/day)			
	0 mg/g/day (control group)	5 mg/g/day	10 mg/g/day	20 mg/g/day
Brain	0.41± 0.02	0.42± 0.02	0.42± 0.03	0.43± 0.02
Heart	0.20± 0.04	0.19± 0.02	0.19± 0.03	0.20± 0.03
Kidneys	0.46± 0.04	0.45± 0.02	0.46± 0.03	0.50± 0.05
Liver	1.58± 0.27	1.60± 0.25	1.56± 0.25	1.61± 0.28
Lung	0.79± 0.03	0.80± 0.04	0.78± 0.03	0.79± 0.03
Pituitary	0.02± 0.01	0.01± 0.00	0.01 ± 0.00	0.01± 0.00
Prostate	0.16± 0.41	0.16± 0.36	0.15 ± 0.35	0.15 ± 0.30
Spleen	0.09± 0.03	0.09± 0.03	0.08± 0.03	0.09± 0.03
Testes	0.19± 0.02	0.19± 0.03	0.18± 0.02	0.20± 0.04
Thymus	0.25± 0.02	0.27± 0.12	0.24± 0.09	0.26 ± 0.10
Parathyroids	0.02± 0.01	0.02± 0.00	0.01± 0.01	0.02 ± 0.01
Epididymis	0.08± 0.01	0.10± 0.02	0.10± 0.01	0.09± 0.02
Adrenal glands	0.06± 0.02	0.07± 0.01	0.06 ± 0.01	0.06 ± 0.01

Data presented as mean ± standard deviation (n = 5), at 5% level of significant ($p<0.05$). Statistical tests: Analysis of variance (ANOVA) followed by Dunnett'S test.

Table 3.7B Organ weight record of female mice (g) at terminal sacrifice (organ to body weight index%).

Organ	Dose (mg/g body weight/day)			
	0 mg/g/day (control group)	5 mg/g/day	10 mg/g/day	20 mg/g/day
Brain	0.43± 0.02	0.44± 0.02	0.44± 0.03	0.45± 0.02
Heart	0.25± 0.04	0.26± 0.02	0.27± 0.03	0.26± 0.03
Kidneys	0.55± 0.04	0.53± 0.02	0.54± 0.03	0.50± 0.05
Liver	1.80± 0.27	1.84± 0.25	1.83± 0.25	1.81± 0.28
Lung	1.01± 2.89	1.08± 2.89	1.21± 2.89	1.00± 2.89
Pituitary	0.03± 0.01	0.03± 0.00	0.04 ± 0.00	0.04± 0.00
Spleen	0.10± 0.03	0.09± 0.03	0.10± 0.03	0.09± 0.03
Thymus	0.37± 0.10	0.35± 0.12	0.40± 0.09	0.39 ± 0.10
Parathyroids	0.03± 0.01	0.03± 0.00	0.03± 0.01	0.03 ± 0.01
Ovaries	0.05± 0.04	0.05± 0.04	0.04± 0.03	0.05 ± 0.03
Uterus with cervix	0.14± 0.07	0.15± 0.05	0.16± 0.06	0.17 ± 0.06
Adrenal glands	0.08± 0.02	0.08± 0.01	0.09 ± 0.01	0.08 ± 0.01

Data presented as mean ± standard deviation (n = 5), at 5% level of significant ($p<0.05$). Statistical tests: Analysis of variance (ANOVA) followed by Dunnett'S test.

- ***Food consumption and water intake***

CMC is widely used food additives and applications are expanding into dietary supplements and additional drug product uses. Determination of consumption of food and intake of water are important parameters in the safety of a product for any therapeutic purpose, as proper intake of nutrients is essential to the physiological status of the animal and to the accomplishment of the proper response to the drugs or chemicals tested [Steven and Mylecrdfaine, 1994; Iversen and Nicolaysen, 2003]. Mean food consumption values were measured on a per cage basis, 5 mice/cage, and expressed as mg food consumed per mice per day, during every weeks of the study. Effect of produced CMC at doses level of 0 (control), 5, 10 and 20 mg/g/day on consumption of food in healthy male and female mice at specific intervals for 90 days are shown in **Figure 3.1** and **Figure 3.2** respectively.

It can be seen from the **Figure 3.1** that for the male mice, the feed consumption values increased from 4.1-4.5 g for control and from 4.05-4.6 g, 4.2-4.65 g and 4.2-4.7 g for 5, 10 and 20 mg/g/day of CMC treated groups. It can also be seen from the **Figure 3.2** that for the female mice, the feed consumption values increased from 4.1-4.5 g for control and from 4.06-4.62 g, 4.22-4.62 g and 4.22-4.7 g for 5, 10 and 20 mg/g/day of CMC treated groups.

Feed consumption values of females administered 5, 10 and 20 mg/g/day of CMC were slightly greater and statistically not significant when compared to control values (**Figure 3.2**) during the study. This was attributed to the need to maintain caloric intake necessary for normal growth as a result of the large amounts of CMC in the diets. Feed consumption values of test compound-treated males fed at 5, 10 and 20 mg/g/day of CMC were slightly comparable to control values as in **Figure 3.1**. The test result indicates that CMC is not genotoxic. Similar result of depolymerized

sodium carboxymethyl cellulose on food consumption of rats has been reported by Bar *et al.* [1995].

Mean water consumption values of both male and female mice were measured on a per cage basis, 5 mice/cage, and expressed as ml water consumed per mice per day, during every weeks of the 90 days study. Effect of extracted CMC at doses level of 0 (control), 5, 10 and 20 mg/g/day on water consumption in healthy male and female mice at specific intervals for 90 days are shown in **Figure 3.3** and **Figure 3.4** respectively. Figures show that water intake tended to increase with increasing dose levels of the test compounds in both male and female mice. Water intake for control male mice was 5.3-5.7 ml/day and increased from 5.32-5.82 ml/day for all treated mice shown in **Figure 3.3**. Also it can be seen from **Figure 3.4** that water intake increased from 5.35-5.85 ml/day for all treated female mice compared to control mice (5.3-5.6 ml/day). Generally a steady increase in the water intake was observed in all the treated mice compared with control. This is because, water intake increased with increasing consumption of dietary food as well as increasing doses of CMC. This finding is supported by the reports of Bar *et al.* [1995] and DeMerlis *et al.* [2005].

There was no significant difference ($P>0.05$) in food consumption, water intake between control and treated groups. The proper intake of nutrients is essential to maintain the physiological state of the animals and to accomplish proper response to plant product tested. In this study, the consumption of food and intake of water were not significantly altered, suggesting that produced product neither induced nor suppressed appetite in healthy mice and had no deleterious effects. Thus this indicates there was no disturbance in carbohydrate, protein or fat metabolism [Attanayake *et al.,* 2015; Klassen, 2001].

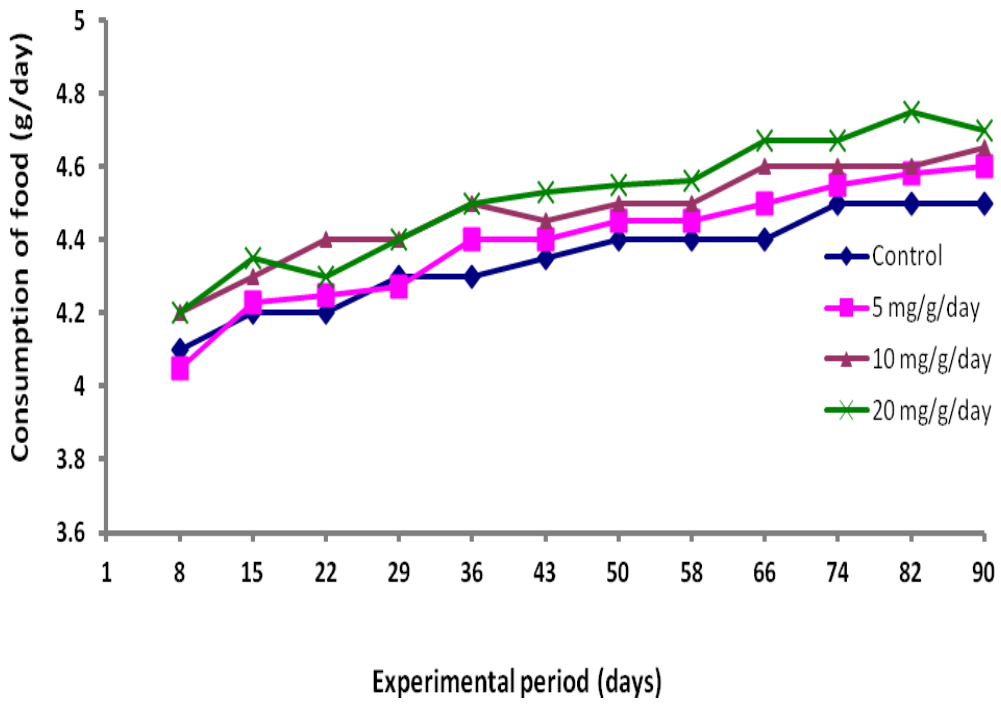

Figure 3.1. Effect of synthesized CMC on consumption of food in healthy male mice at specific intervals for 90 days. The values are expressed as mean ± standard error of mean (n = 5/group). The two sample t-test at á = 0.05 showed no statistical difference between consumption of food in product treated mice compared to untreated healthy control mice.

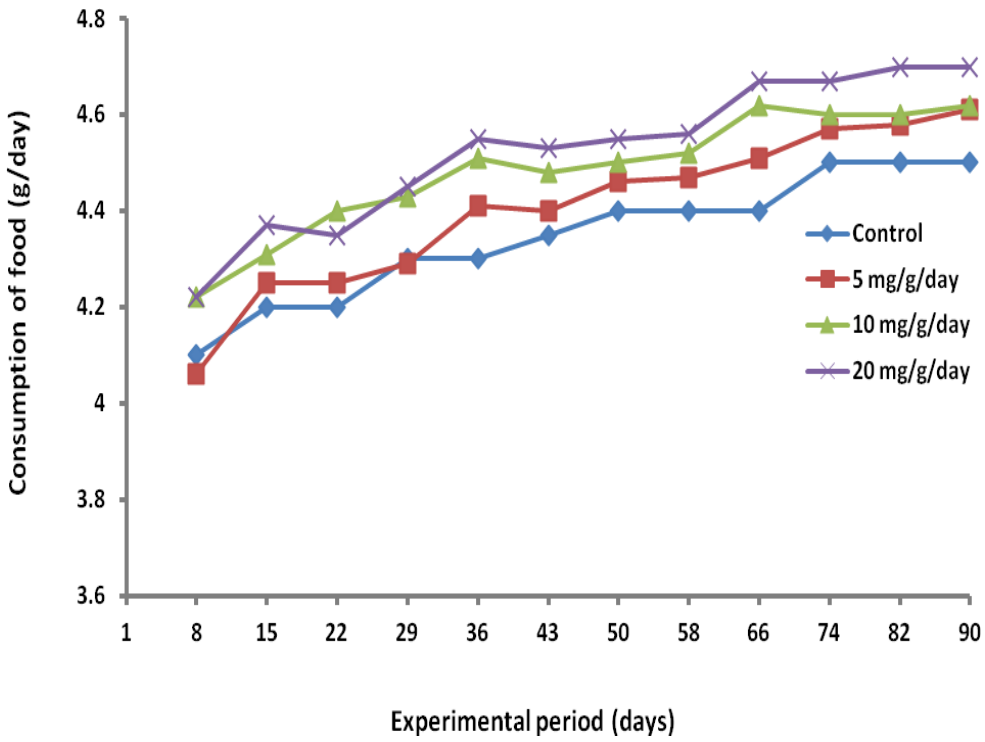

Figure 3.2. Effect of synthesized CMC on consumption of food in healthy female mice at specific intervals for 90 days. The values are expressed as mean ± standard error of mean (n = 5/group). The two sample t-test at á = 0.05 showed no statistical difference between consumption of food in product treated mice compared to untreated healthy control mice.

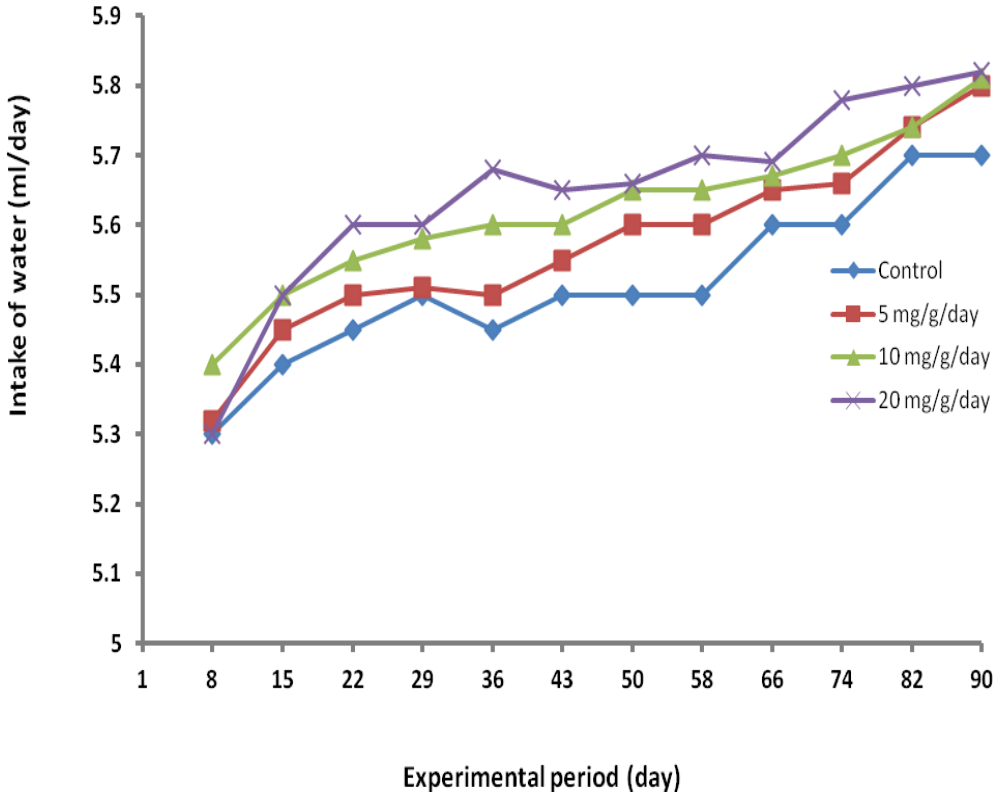

Figure 3.3. Effect of synthesized CMC on intake of water in healthy male mice at specific intervals for 90 days. The values are expressed as mean ± standard error of mean (n = 5/group). The two sample t-test at á = 0.05 showed no statistical difference between intake of water in CMC treated mice compared to untreated healthy control mice.

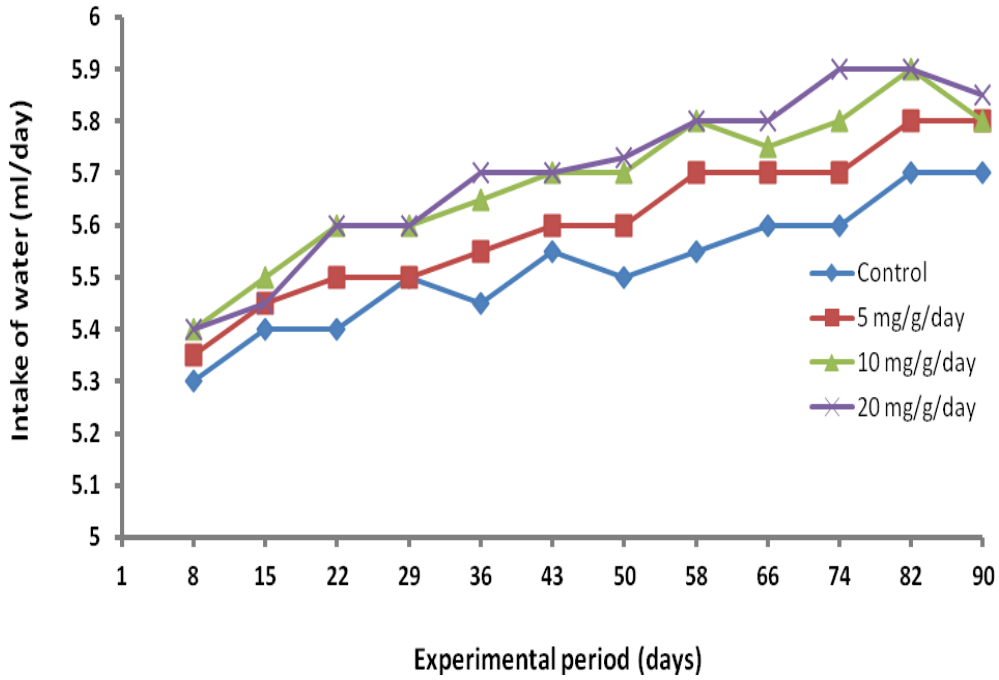

Figure 3.4. Effect of synthesized CMC on intake of water in healthy female mice at specific intervals for 90 days. The values are expressed as mean ± standard error of mean (n = 5/group). The two sample t-test at á = 0.05 showed no statistical difference between intake of water in CMC treated mice compared to untreated healthy control mice.

- ***Urinalysis***

Urinalysis is primarily concerned with kidney function and is valuable in the identification of chemically induced renal toxicity but can also provide information about the functioning of organ system outside the urinary tract. Urinalysis is a useful tool for accessing a longitudinal effect of a test substance by repetitive sampling [Gwon *et al.*, 2009; Yamada, 2009]. Urinalysis data are indicators of kidney disease, but they are frequently affected by stress due to environmental changes such as oliguria or contamination with feces or pellets [Hayes, 2001; Meyer and Harvey, 1998].

Mean urine volume (measured on a per cage basis, 5 mice/cage, and expressed as ml urine excretion per mice per day) was collected monthly during the study. Then urine samples from mice were analyzed by an auto analyzer (FA-100) for specific gravity, pH, sodium, potassium, chloride and citrate concentration of urine. The results of urinary analysis of both male and female mice administered orally CMC for 90 days are shown in **Table 3.8A** and **Table 3.8B** respectively. Some significant changes were observed and are described below.

In line with the dose-related increase of water consumption, urine volume also increased with increasing doses of CMC in male (1.0-1.18 ml/day) and female mice (1.15-1.30 ml/day) compared to control mice (0.8 ml/day for male and 0.9 ml/day for female mice) during the 24 h urine collection period. Urine volume of treated female mice was higher than that of male mice. These differences were increasing consumption of diet and gender related. Urine densities were decreased in a dose–responsive manner, while urinary volume as well as CMC dose levels increased. Decreases in urinary concentrations of several parameters were a result of dilutional, related to increased urinary volume which was supported by the reports of James *et al*. [2007].

Urinary pH of CMC treated groups (7.67-8.80 for male and 8.0-9.0 for female mice) increased above control groups (7.1) due to 24 h urinary excretion of sodium, calcium and citrate. Sodium excretion increased with increasing percentage of CMC-dose level in both male and female mice. CMC is a sodium salt. A high consumption of sodium salts of weak or moderate acids is associated with an increase in urinary pH [Degroot *et al.,* 1989, Fukushima *et al.,* 1986a;b; Shibata *et al.,* 1989]. The data of the present study also show that CMC exert a similar alkalizing effect.

Potassium, chloride and citrate percentages increased in high-dose CMC fed animal which was direct consequences of higher sodium intake. These effects can be attributing to the ingestion of sodium. Many studies in rat and mice have shown that the ingestion of low digestible carbohydrates (e.g. lactose, modified starches) and polyols causes an increased citrate excretion by a mechanism that is not yet completely understood [Bar, 1985; Lord and Newberne, 1990; Newberne *et al*., 1988; Roe, 1989]. Increases in the excretion of citrate and, less consistently, calcium were seen in the present study also in response to the ingestion of CMC. Though, ingestion of food additive was well tolerated [Bar *et al.*, 1995].

There were occasional statistically significant and apparently dose related effects noted (for example, sodium, chloride, and citrate), but in the absence of functional changes and histopathological findings these effects were, therefore, not considered toxicologically or biologically significant [DeMarlis *et al.,* 2005].

Table 3.8A Effect of daily administration of CMC on urinary parameters for male mice at every month of the experiment.

Days	Parameters	Dose (mg/g body weight/day)			
		Control	**5**	**10**	**20**
30	Volume ml/24 h	0.8±0.07	1.0± 0.70	1.10±0.33	1.18±0.97
	Density kg/L	1.045±0.53	1.044±0.53	1.044±0.53	1.043±0.53
	pH	7.1±0.90	7.67±0.80	8.1±0.90	8.80±0.20
	Na mEq/L	136.0±1.70	139.80±3.00	141.00±2.70	143.40±2.40
	K mEq/L	6.0±0.70	6.06±.30	6.01±0.70	6.40±0.40
	Cl^- mEq/L	88.0±5.90	89.0±5.90	110.0±5.90	107.70±4.30
	Ca mg/dL	9.06±0.41	9.30±0.24	9.42±0.28	9.50±0.21
60	Volume ml/24 h	0.9±0.07	1.12± 0.70	1.20±0.33	1.25±0.97
	Density kg/L	1.045±0.53	1.043±0.53	1.044±0.53	1.042±0.53
	pH	7.2±0.90	7.67±0.80	8.1±0.90	8.80±0.20
	Na mEq/L	139.0±1.70	141.80±3.00	143.00±2.70	145.40±2.40
	K mEq/L	6.0±0.70	6.10±0.30	6.00±0.70	6.40±0.40
	Cl^- mEq/L	88.80±3.50	95.30±4.80	90.80±3.50	107.70±4.30
	Ca mg/dL	9.21±0.41	9.56±0.24	10.12±0.28	10.11±5.90
90	Volume ml/24 h	0.9±0.07	1.20± 0.70	1.28±0.33	1.30±0.97
	Density kg/L	1.045±0.53	1.044±0.53	1.043±0.53	1.042±0.53
	pH	7.50±0.90	7.67±0.80	8.1±0.90	8.80±0.20
	Na mEq/L	139.0±1.70	141.80±3.00	143.00±2.70	146.40±2.40
	K mEq/L	6.0±0.70	6.50±0.30	6.51±0.70	6.63±0.40
	Cl^- mEq/L	90.0±5.90	92.30±4.80	91.80±3.50	110.70±4.30
	Ca mg/dL	9.88±0.21	9.06±0.24	10.12±0.28	10.28±0.21

Value presented as mean ± standard deviation (n = 5 mice/group/sex), at 5% level of significant ($p<0.05$). Statistical tests: ANOVA followed by Dunnett's tests.

Table 3.8B Effect of daily administration of CMC on urinary parameters for female mice at every month of the experiment.

Days	Parameters	Dose (mg/g body weight/day)			
		Control	**5**	**10**	**20**
30	Volume ml/24 h	0.90±0.07	1.15± 0.70	1.27 ±0.33	1.30±0.97
	Density kg/L	1.045±0.53	1.043±0.53	1.042±0.53	1.040±0.53
	pH	7.1±0.90	8.0±0.80	8.80±0.90	9.00±0.20
	Na mEq/L	137.0±1.70	142.80±3.00	144.00±2.70	148.40±2.40
	K mEq/L	6.0±0.70	6.30±0.30	6.35±0.70	6.40±0.40
	Cl^- mEq/L	89.80±3.50	94.30±4.80	90.80±3.50	100.70±4.30
	Ca mg/dL	9.95±0.21	9.96±0.24	9.95±0.28	9.98±0.21
60	Volume ml/24 h	0.9±0.07	1.23± 0.70	1.27±0.33	1.30±0.97
	Density kg/L	1.045±0.53	1.043±0.53	1.042±0.53	1.041±0.53
	pH	7.2±0.90	7.97±0.80	8.81±0.90	9.01±0.20
	Na mEq/L	138.0±1.70	143.80±3.00	145.00±2.70	146.40±2.40
	K mEq/L	6.0±0.70	6.35±.30	6.40±0.70	6.41±0.40
	Cl^- mEq/L	90.0±5.90	92.30±4.80	98.80±3.50	107.70±4.30
	Ca mg/dL	9.97±0.21	9.85±0.24	10.00±0.28	10.08±0.21
90	Volume ml/24 h	0.9±0.07	1.29± 0.70	1.30±0.33	1.31±0.97
	Density kg/L	1.045±0.53	1.043±0.53	1.042±0.53	1.040±0.53
	pH	7.2±0.90	8.01±0.80	8.71±0.90	8.99±0.20
	Na mEq/L	139.0±1.70	142.80±3.00	146.00±2.70	147.40±2.40
	K mEq/L	6.0±0.70	6.08±.30	6.30±0.70	6.50±0.40
	Cl^- mEq/L	90.0±5.90	91.30±4.80	99.80±3.50	110.70±4.30
	Ca mg/dL	9.96±0.24	9.98±0.24	10.02±0.28	9.98±0.21

Value presented as mean ± standard deviation (n = 5 mice/group/sex), at 5% level of significant ($p<0.05$).
Statistical tests: ANOVA followed by Dunnett's tests.

- ***Haematological parameters***

Haematological parameters are a valuable tool for assessing the injures that caused by certain substances [Al-Shinnawy, 2009]. The assessment of haematological parameters can be used to determine the extent of deleterious effects of foreign compounds on blood constituents of an animal. The hematopoietic system is very sensitive to toxic compounds and serves as an important index of the physiological and pathological status. [Kulkarni and Veeranjaneyulu, 2010; Adeneye *et al*., 2006]. Such toxicity testing is relevant for changes in the hemopoietic system that has a higher predictive value for human toxicity, when extrapolated from animal studies.

The results of haematological data of both mice are shown in **Table 3.9A** and **3.9B**. Sub-chronic exposure of albino mice to the agrowaste product produced small and transient changes in haematological parameter, e.g., red blood cells count (RBC), platelet count, WBS and its differentials. There was increase in RBC concentration in the test groups compared to the control group. But, the values were statistically not significant ($P > 0.05$). Therefore, the results revealed that the maize husk derived CMC was not toxic to the circulatory red blood cells, and did not interfere with their production. Therefore, it is plausible to assume the CMC is not hematotoxic. This observation was in agreement with Kumar and Sastry [2010] and Attanayaka *et al*. [2015].

Platelets were increased in female mice of all groups ($p<0.05$) whereas platelets decreased in male mice of all groups ($p<0.05$ or $p<0.01$) compared to control groups. Lymphocyte was increased in male and female mice in the 5 mg/g/day CMC groups (<0.05). Although monocytes were decreased in the 5 and 10 mg/g/day CMC groups, it was decreased slightly in male mice and more significantly in female mice

($p<0.05$). There were also no significant differences in hemoglobin concentration at all dose levels compared to control level while hemoglobin concentrations were slightly higher in male mice than that of female mice that was not dose dependent. In the differential leukocyte count, neutrophils showed significant difference at the dose levels of 10 and 20 mg/g compared to the values at the dose of 5 mg/g and control. All differences of these parameters were considered to be fortuitous since they were not dose related, occurred in one sex only or were numerically very small.

Inflammatory process is characterized by the involvement of multiple inflammatory cells of the white blood cells, WBC [Kytridis and Manetas, 2006]. Even though there were minor alterations in the percentage of white blood cells, the assessment of histopathology of the liver did not exhibit any abnormalities in mice treated with the extracted product, implying absence of any abnormal effects on hematopoiesis and immunologic functions [Anoja *et al.,* 2015].

Table 3.9A Effect of daily administration of CMC on haematological parameters for male mice at every month of the experiment.

Days	Parameters	Dose (mg/g body weight/day)			
		Control	**5**	**10**	**20**
30	White blood cell ($\times 10^9$/L)	9.39±3.07	10.88± 1.70	11.01±3.33	9.07±2.97
	Red blood cell ($\times 10^{12}$/L)	6.88±0.53	7.06±0.20	6.95±0.45	7.04±0.35
	Platelet ($\times 10^{12}$/L)	1017.80±108.20	1010.0±122.80	1006.80±247.80	988.30±141.90
	Hemoglobin(g/dL)	10.60±0.90	11.09±1.30	11.58±1.00	11.70±1.40
	Lymphocytes (%)	77.50±5.90	80.30±4.80	78.00±3.50	77.70±4.30
	Neutrophils (%)	23.20±1.90	24.70±1.60	32.30±0.90	30.80±1.10
	Monocyte (%)	5.00±1.20	4.97±0.60	4.89±0.90	5.10±1.00
60	White blood cell ($\times 10^9$/L)	10.03±2.17	10.80± 1.50	9.01±3.03	11.07±2.90
	Red blood cell ($\times 10^{12}$/L)	6.80±0.59	7.00±0.10	6.98±0.75	7.05±0.55
	Platelet ($\times 10^{12}$/L)	1015.80±108.20	1012.00±122.80	1006.80±247.80	990.30±141.90
	Hemoglobin(g/dL)	11.60±0.91	10.00±1.03	11.09±1.01	10.90±1.40
	Lymphocytes (%)	79.60±4.90	81.30±3.80	78.80±3.50	78.70±3.30
	Neutrophils (%)	25.20±1.80	21.70±1.60	29.30±0.90	31.80±1.11
	Monocyte (%)	4.67±1.20	4.55±0.65	4.60±0.70	5.01±1.09
90	White blood cell ($\times 10^9$/L)	9.39±2.77	10.08± 1.71	11.01±2.33	10.03±1.97
	Red blood cell ($\times 10^{12}$/L)	6.99±0.55	7.03±0.22	7.00±0.80	7.04±0.30
	Platelet ($\times 10^{12}$/L)	1020.80±108.20	1016. ±122.80	1016.80±247.80	999.30±141.90
	Hemoglobin(g/dL)	10.60±0.90	10.90±1.30	11.09±1.00	10.90±1.40
	Lymphocytes (%)	77.59±5.80	82.30±4.80	70.80±3.10	77.10±3.30
	Neutrophils (%)	26.20±0.90	25.70±1.61	34.29±0.70	33.70±1.09
	Monocyte (%)	5.06±1.20	4.99±0.60	5.00±0.60	5.11±1.11

Value presented as mean ± standard deviation (n = 5 mice/group/sex), at 5% level of significant ($p<0.05$). Statistical tests: ANOVA followed by Dunnett's tests.

Table 3.9B Effect of daily administration of CMC on haematological parameters for female mice at every month of the experiment.

Days	Parameters	Dose (mg/g body weight/day)			
		Control	**5**	**10**	**20**
30	White blood cell ($\times 10^9$/L)	9.01±3.07	10.22± 1.70	10.01±3.33	9.07±2.97
	Red blood cell ($\times 10^{12}$/L)	6.80±0.53	7.04±0.20	6.99±0.45	7.05±0.35
	Platelet ($\times 10^{12}$/L)	983.30±141.9	1019.±122.80	1046.80±247.80	1018.80±108.20
	Hemoglobin(g/dL)	9.90±0.90	9.68±1.30	10.09±1.00	9.79±1.40
	Lymphocytes (%)	77.50±5.90	80.30±4.80	75.80±3.50	76.70±4.30
	Neutrophils (%)	22.20±1.90	23.70±1.60	29.30±0.90	30.80±1.10
	Monocyte (%)	5.20±1.20	4.70±0.60	4.80±0.90	5.10±1.00
60	White blood cell ($\times 10^9$/L)	10.03±2.17	10.70± 1.50	9.01±3.03	11.02±2.90
	Red blood cell ($\times 10^{12}$/L)	6.80±0.59	7.00±0.10	6.98±0.75	7.05±0.55
	Platelet ($\times 10^{12}$/L)	989.30±141.90	1029. ±122.80	1037.80±247.80	1040.80±108.20
	Hemoglobin(g/dL)	10.06±0.91	10.00±1.03	9.09±1.01	10.01±1.40
	Lymphocytes (%)	79.60±4.90	81.30±3.80	78.80±3.50	80.70±3.30
	Neutrophils (%)	25.20±1.80	24.70±1.60	31.30±0.90	31.80±1.11
	Monocyte (%)	5.10±1.20	4.33±0.65	4.21±0.70	5.22±1.09
90	White blood cell ($\times 10^9$/L)	10.39±2.77	10.08± 1.71	11.00±2.33	10.03±1.97
	Red blood cell ($\times 10^{12}$/L)	6.90±0.55	7.02±0.22	7.03±0.80	7.03±0.30
	Platelet ($\times 10^{12}$/L)	1000.30±141.90	1009. ±122.80	1009.80±247.80	1047.80±108.20
	Hemoglobin(g/dL)	9.62±0.90	10.50±1.30	10.10±1.00	9.90±1.40
	Lymphocytes (%)	75.59±5.80	82.30±4.80	70.80±3.10	77.10±3.30
	Neutrophils (%)	28.20±0.90	28.70±1.61	32.99±0.70	32.70±1.09
	Monocyte (%)	5.60±1.20	4.09±0.60	4.80±0.90	5.61±1.11

Value presented as mean ± standard deviation (n = 5 mice/group/sex), at 5% level of significant ($p<0.05$). Statistical tests: ANOVA followed by Dunnett's tests.

- ***Assessment of biochemical parameters***

Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells. This interaction may vary depending on the chemical properties of the toxicants and the cell membrane, as it may occur on the cell surface, within the cell body, or in the tissues beneath as well as at the extracellular matrix. The toxic effects may take place prior to the binding of the toxicants to the vital organs such as liver and kidneys [Asante-Duah, 2002].

Liver, kidney and heart plays an important role in the detoxification of toxic substances *in vivo*. Considering the key role in detoxification of natural toxic substances, the liver was selected to conduct a detailed assessment of biochemical parameters and histology on hematoxylin and eosin stained sections [Anoja *et al.,* 2015]. Liver cell damage is characterized by a rise in hepatic enzymes like ALT, AST and ALP [Abeywickrama *et al.,* 2011; Adam, 1998 and Hayes, 1989]. AST and ALT are released in the heart and an elevation in their plasma concentrations are indicators of cardiac damage [Mythilpriya *et al.,* 2007; Crook, 2006].

The effects of CMC at different dose levels on biological parameter of male and female mice are shown in **Table 3.10A** and **3.10B**. The activity of aspartate amino transferase (AST) showed no significant change in all dose levels studied. At doses of 5 and 10 mg/g body weight compared to the control, there was also no significant change in the activity of alanin amino transferase (ALT). However, a significant increase was observed at the dose of 20 mg/g in males than in controls. The activity of plasma alkaline phosphatase (ALP) increased at doses of 20 mg/g, but no significant difference was observed at lower doses of 5 mg/g and 10 mg/g compared to the control. Since in this study the enzymes showed no appreciable

increase or decrease in the treated animals, it implies that the product has no hepato-toxic effect. This was confirmed by the histological study in which tissue morphology showed no changes. These results were supported by reports in the literature [Govindwar and Dalvi, 1990; Mbaka *et al.,* 2010; Abdel-Rahim *et al.,* 1989]

The transaminases (SGOT and SGPT) are also well known enzymes used as biomarkers predicting possible toxicity [Rahman *et al*., 2001]. Generally, damage to the parenchymal liver cells will result in elevations of both these transaminases [Wolf *et al.,* 1972]. High levels of SGOT indicate the loss of functional integrity of liver, such as that of viral hepatitis, as well as cardiac infraction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Liver toxicants cause disturbances in synthesis and metabolism of triglycerides, cholesterol and lipoproteins, the increased triglyceride content in the blood is in correlation with the fatty degeneration of the liver.

In the present study, the transaminases were not significantly increased at all dose levels of CMC compared to control for both male and female mice. It Suggests that chronic ingestion of CMC did not alter the hepatocytes and kidneys of the mice, and, furthermore the normal metabolism of the animals. This finding was in agreement with other researcher [Kumar and Sastry, 2010].

Urea and creatinine are considered as a suitable prognostic indicator of renal dysfunction and kidney failure for any toxic compounds [Gnanamani *et al*., 2008]. In this study, most of the renal parameters i.e creatinine, Bilirubin, urea, were unchanged by the doses of 5, 10 and 20 mg/g CMC as compared with the control, throughout the total experimental period which is the good indicator of kidney functions [Hilaly *et al.,* 2004]. The lack of significant alterations in the levels of creatinine, bilirubin, and

urea, after the days 30, 60 and 90 means that CMC has no harmful effect on the kidney.

In the biochemical analysis performed as part of this study, dietary CMC significantly increased the serum glucose and serum cholesterol values compared to control, and significantly decreased the serum triglyceride values. However, these values remained within the normal range throughout the experimental period, indicating that there was no CMC toxicity.

Table 3.10A Effect of daily administration of CMC on biochemical parameters for male mice at every month of the experiment.

Days	Parameters	Dose (mg/g body weight/day)			
		Control	5	10	20
30	Serum glucose (mg/dL)	120.39±3.07	140.88± 1.70	130.01±3.33	145.07±2.97
	Serum cholesterol (mg/dL)	185.88±1.53	200.06±2.20	194.95±1.45	210.04±2.35
	Serum urea (mg/dL)	36.30±0.90	25 ±1.00	28.80±0.80	29.10±1.20
	Serum creatinine (mg/dL)	66.30±0.90	70 ±1.00	69.80±0.80	67.10±1.20
	Serum triglyceride (mg/dL)	105.60±1.70	112.80±1.30	120.00±1.70	99.40±1.40
	Serum bilirubin (mg/dL)	0.90±0.60	0.99±0.80	1.10±0.50	1.00±0.30
	ALT (U/L)	52.20±1.90	56.70±1.60	59.34±1.90	73.80±3.50
	AST (U/L)	163.90±0.11	162.70±0.12	165.11±0.13	164.10±0.10
	ALP (U/L)	130.20±1.90	120.70±1.60	135.30±1.90	139.80±1.50
	SGOT (U/L)	69.30±1.00	71.00±1.20	70.00±1.40	75.50±1.40
	SGPT (U/L)	75.30±1.10	81.00±1.20	79.00±1.80	80.50±1.90
60	Serum glucose (mg/dL)	122.09±2.07	145.80± 1.90	135.01±2.30	140.08±2.07
	Serum cholesterol (mg/dL)	188.88±1.07	206.06±1.60	190.95±1.45	200.04±2.30
	Serum urea (mg/dL)	30.10±0.98	29 ±1.07	28.80±0.80	29.11±1.22
	Serum creatinine (mg/dL)	67.30±0.90	71 ±1.00	69.80±0.80	68.10±1.20
	Serum triglyceride (mg/dL)	105.60±0.99	112.80±1.30	120.00±1.70	100.41±1.34
	Serum bilirubin (mg/dL)	0.99±0.50	0.99±0.90	1.11±0.62	1.09±0.36
	ALT (U/L)	54.10±1.90	56.71±1.60	59.34±1.90	72.81±3.50
	AST (U/L)	164.95±0.17	163.70±0.11	165.11±0.13	163.13±0.17
	ALP (U/L)	135.10±1.20	130.40±1.60	130.30±1.80	142.80±1.00
	SGOT (U/L)	70.30±1.00	71.00±1.20	70.00±1.40	72.50±1.40
	SGPT (U/L)	75.30±1.10	81.00±1.20	79.00±1.80	71.50±1.90
90	Serum glucose (mg/dL)	121.39±3.07	143.80± 1.70	136.01±3.03	146.07±2.07
	Serum cholesterol (mg/dL)	180.80±1.60	201.06±2.10	190.85±1.05	209.04±2.30
	Serum urea (mg/dL)	29.40±0.91	28 ±1.01	28.90±0.90	30.11±1.22
	Serum creatinine (mg/dL)	69.30±0.90	70 ±1.00	67.80±0.80	67.10±1.20
	Serum tryglyceride(mg/dL)	105.50±1.80	110.80±1.30	100.00±1.70	99.10±1.33
	Serum bilirubin (mg/dL)	0.99±0.70	0.98±0.83	1.11±0.51	1.02±0.37
	ALT (U/L)	52.20±1.90	56.70±1.60	59.34±1.90	71.80±3.50
	AST (U/L)	160.95±0.11	163.70±0.11	161.10±0.13	162.10±0.10
	ALP (U/L)	131.20±1.41	131.70±1.40	130.33±0.90	139.70±1.05
	SGOT (U/L)	69.30±1.00	71.02±1.20	71.00±1.40	76.50±1.00
	SGPT (U/L)	74.30±1.10	80.00±1.20	79.00±1.80	80.50±1.90

Values are expressed as mean ± S.E.M of 5 animals (one-way ANOVA); $p>0.05$ vs control group (student t-test). Significantly different from control, $p<0.05$.

Table 3.10B Effect of daily administration of CMC on biochemical parameters for female mice at every month of the experiment.

Days	Parameters	Dose (mg/g body weight/day)			
		Control	5	10	20
30	Serum glucose (mg/dL)	120.39±3.07	140.88± 1.70	130.01±3.33	145.07±2.97
	Serum cholesterol (mg/dL)	185.88±1.53	200.06±2.20	194.95±1.45	210.04±2.35
	Serum urea (mg/dL)	33.30±0.90	35 ±1.00	32.80±0.80	34.10±1.20
	Serum creatinine (mg/dL)	68.30±0.90	70 ±1.00	69.80±0.80	67.10±1.20
	Serum triglyceride (mg/dL)	105.60±1.70	112.80±1.30	120.00±1.70	99.40±1.40
	Serum bilirubin (mg/dL)	0.95±0.60	0.98±0.80	1.01±0.50	1.00±0.30
	ALT (U/L)	52.20±1.90	56.70±1.60	59.34±1.90	60.80±3.50
	AST (U/L)	161.95±0.11	163.70±0.11	161.10±0.10	162.10±0.10
	ALP (U/L)	130.20±1.90	120.70±1.60	135.30±1.90	139.80±1.50
	SGOT (U/L)	69.30±1.00	72.00±1.20	71.00±1.4	69.50±1.40
	SGPT (U/L)	75.30±1.10	81.00±1.20	79.00±1.80	80.50±1.90
60	Serum glucose (mg/dL)	122.09±2.07	145.80± 1.90	135.01±2.30	140.08±2.07
	Serum cholesterol (mg/dL)	188.88±1.07	206.06±1.60	190.95±1.45	200.04±2.30
	Serum urea (mg/dL)	29.30±0.98	27 ±1.07	28.80±0.80	29.11±1.22
	Serum creatinine (mg/dL)	66.30±0.90	70 ±1.00	68.80±0.80	67.10±1.20
	Serum triglyceride (mg/dL)	105.60±0.99	112.80±1.30	120.00±1.70	100.41±1.34
	Serum bilirubin (mg/dL)	1.00±0.50	0.99±0.90	1.10±0.62	1.09±0.36
	ALT (U/L)	50.20±1.09	53.70±1.10	58.34±1.90	54.80±3.50
	AST (U/L)	160.05±0.11	160.71±0.11	161.10±0.13	161.11±0.10
	ALP (U/L)	132.10±1.20	130.40±1.60	131.30±1.80	143.80±1.00
	SGOT (U/L)	70.30±1.00	71.00±1.20	70.00±1.40	80.50±1.40
	SGPT (U/L)	77.30±1.10	81.00±1.20	79.00±1.80	80.50±1.90
90	Serum glucose (mg/dL)	121.39±3.07	143.80± 1.70	136.01±3.03	146.07±2.07
	Serum cholesterol (mg/dL)	180.80±1.60	201.06±2.10	190.85±1.05	209.04±2.30
	Serum urea (mg/dL)	37.40±0.91	36 ±1.01	38.90±0.90	35.11±1.22
	Serum creatinine (mg/dL)	65.30±0.90	70 ±1.00	67.80±0.80	66.10±1.20
	Serum triglyceride (mg/dL)	105.50±1.80	110.80±1.30	100.00±1.70	99.10±1.33
	Serum bilirubin (mg/dL)	0.98±0.70	0.98±0.83	1.01±0.51	1.21±0.37
	ALT (U/L)	52.20±1.90	54.70±1.60	53.34±1.90	52.80±3.50
	AST (U/L)	159.05±0.11	160.71±0.11	160.10±0.13	161.11±0.10
	ALP (U/L)	132.20±1.41	131.70±1.40	134.33±0.90	139.70±1.05
	SGOT (U/L)	69.30±1.00	71.02±1.20	71.00±1.40	86.50±1.00
	SGPT (U/L)	76.30±1.10	79.00±1.20	78.00±1.80	80.50±1.90

Values are expressed as mean ± S.E.M of 5 animals (one-way ANOVA); $p>0.05$ vs. control group (student t-test). Significantly different from control, $p<0.05$.

- ***Histopathological examination***

The assessment of histopathology in the body tissues is the gold standard for evaluating treatment-related pathological changes in tissues [Jothy *et al.,* 2011]. The evaluation of adverse effects of sub-chronic oral dosing based on histological analysis in experimental animal may be more relevant in determining the overall toxicity of the compound. The liver, heart, kidney and lungs are the primary organs affected by metabolic reaction caused by toxicants [Ekeanyanwu and Njoku , 2014].

Histopathological alterations have been used as markers to better understand animal health exposed to contaminants in lab [Wester and Canton, 1991] and field studies [Hinton *et al.,* 1992; Schwaiger *et al.,* 1997; Teh *et al.,* 1997]. The main advantages of using histopathological markers in monitoring is that this category of markers allows to study the target organs, including brain, heart, lung, kidney and liver, that are responsible for important functions, such as excretion and the deposition and bio-magnifications of toxins in the animal [Gernhofer *et al.,* 2001]. The changes found in these organs are normally as general to pinpoint than functional ones [Fanta *et al.,* 2003], and exhibit as alarming signals of alterations to general animal health [Hinton and Lauren, 1990].

The histopathological observations of the liver, kidney, heart and lung of mice treated with highest doses of CMC are presented in **Table 3.11**. In the present study, histopathological evaluation of repeated dose 90-days treatment indicated that the produced waste product did not adversely affect heart, lung, liver and kidney of mice which corroborated the results of biochemical analysis. Most of the observed statistically non significant variations were random, unrelated to dose and unaccompanied by any histological correlations.

Table 3.11 Histopathological examinations of control and experimental mice (at highest dose level) after the end of the experiment.

Name of experiment	Dose mg/g/day	Histopathological changes observed			
		Liver	Kidney	Heart	Lung
Control	Normal diet	NAD	NAD	NAD	NAD
Test compound	20	NAD	NAD	NAD	NAD

NAD = No Abnormality Detected

The transverse sections of all organs were viewed under the light microscope using multiple magnification power and their histopathological photographs were presented in the **Figures 3.5-3.8.**

The histological features of liver for control and treated mice with highest doses of CMC obtained from this study are displayed in **Figure 3.5a** and **3.5b** respectively and marked with vein and kuppfer cell. The morphology of liver cell in both control and treated groups were normal and no structural damages were observed. Histopathology of the liver sections of the treated mice showed normal hepatic architecture and normal liver lobular structure with well-preserved cytoplasm, prominent nucleus and nucleolus compared with control mice.

The kidney micrograph of control and treated mice displayed in **Figure 3.6a** and **3.6b** respectively. No adverse effects were observed in both groups and the glomeruli and capsules appeared normal and the Bowman's space are also marked clearly.

In addition, the morphological changes of both control and treated mice (**Figure 3.7a** and **3.7b**) in heart tissue were assessed on light microscopy to evaluate

the toxic effects of product on kidney. Age-related naturally-occurring lesions were observed in the tissues, but no abnormalities or damage due to the ingestion of CMC was observed.

Furthermore, in this study the microscopic inspection of the lung (**Figure 3.8a**) of the mice treated with CMC did not indicate any changes in the organs compared to control mice (**Figure 3.8b**).

Macroscopic examination of all organs of the animals treated with waste product showed no changes in colour compared to control. Autopsy at the end of the experiment period revealed no apparent changes. The microscopic examination revealed that, all the organs from the product treated mice did not show any alteration in cell structure or any unfavourable effects and there were no congestion of blood vessels, cellular inflammation, stromal oedema and necrosis.

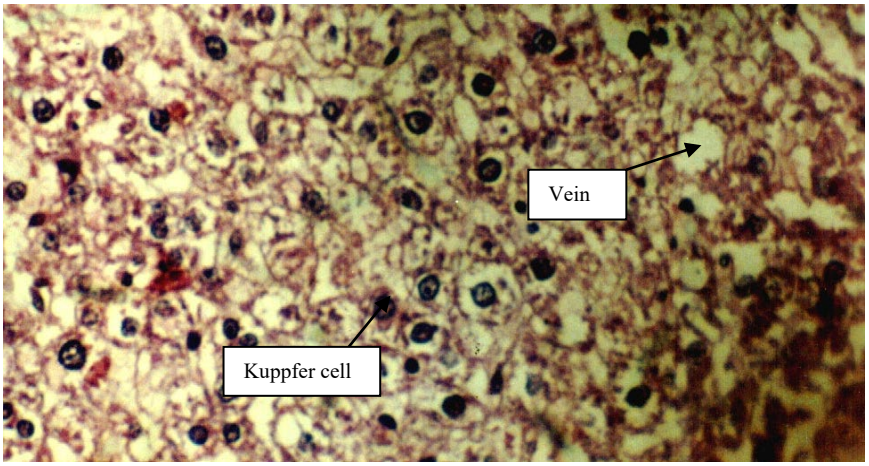

Figure 3.5a. Microphotograph of Liver (control) at HE × 100

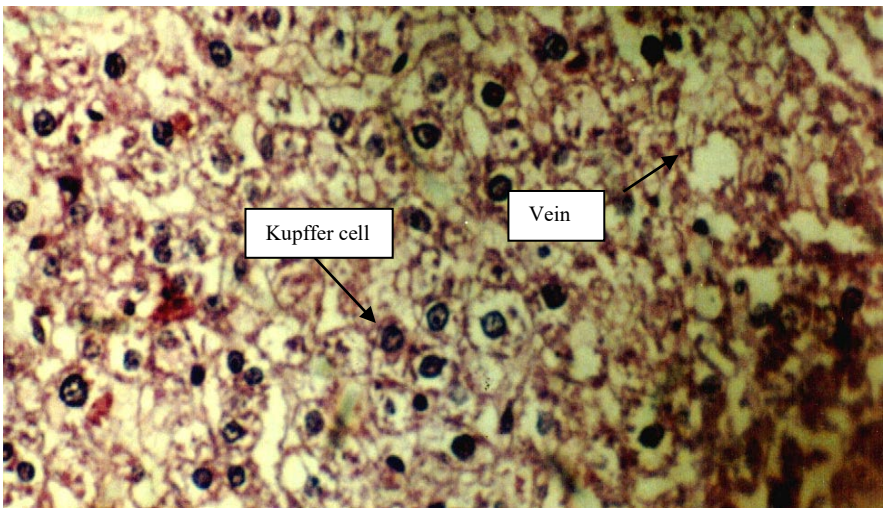

Figure 3.5b. Microphotograph of Liver (treated) at HE × 100

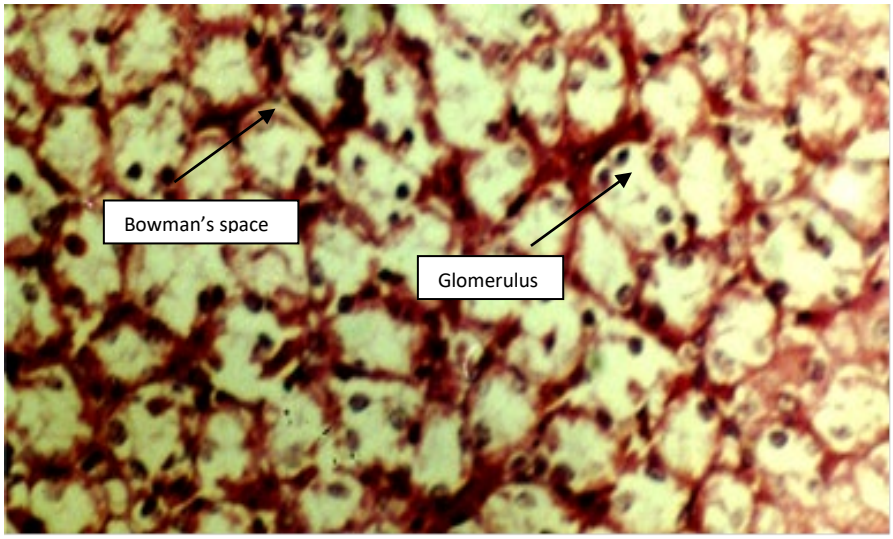

Figure 3.6a Microphotograph of Kidney (control) at HE × 100

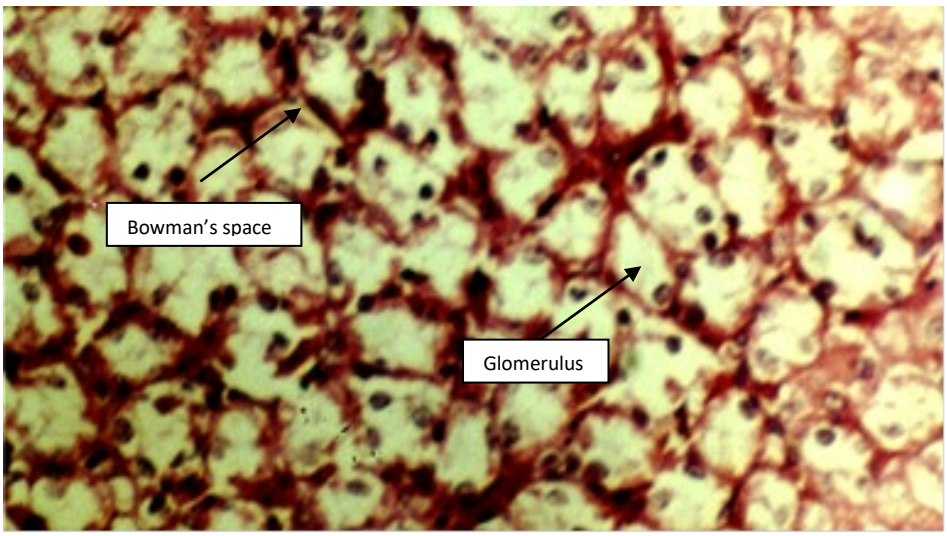

Figure 3.6b Microphotograph of Kidney (treated) at HE × 100

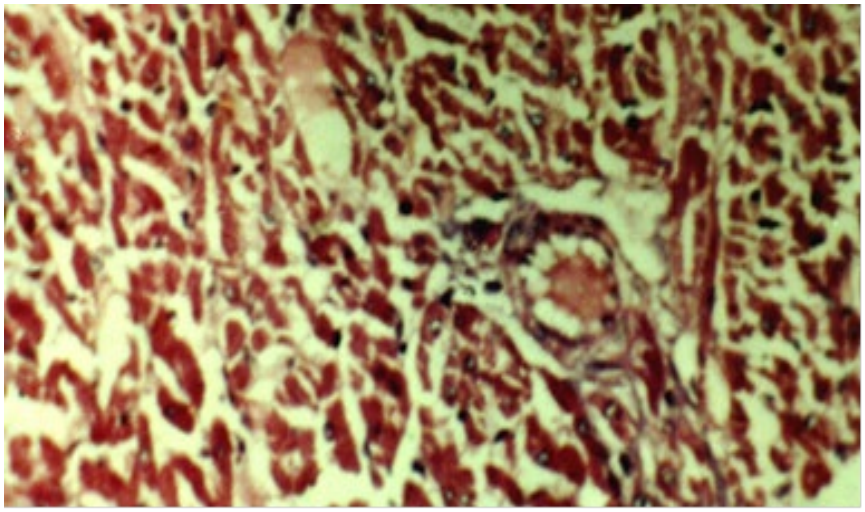

Figure 3.7a Microphotograph of Heart (control) at HE × 100

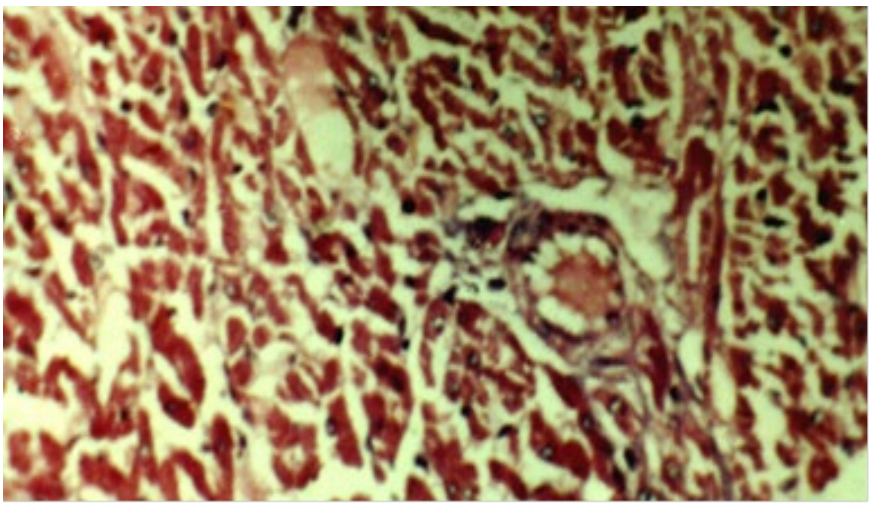

Figure 3.7b Microphotograph of Heart (treated) at HE × 100

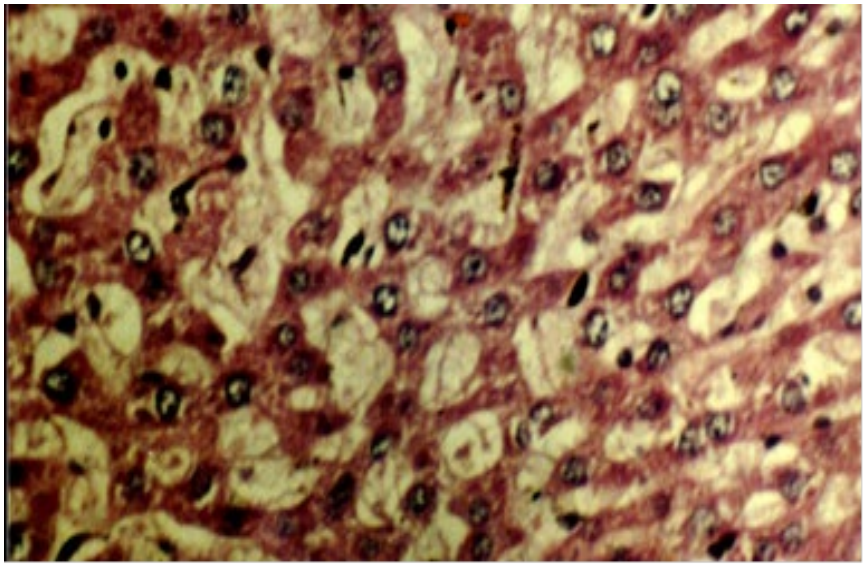

Figure 3.8a Microphotograph of Lung (control) at HE × 100

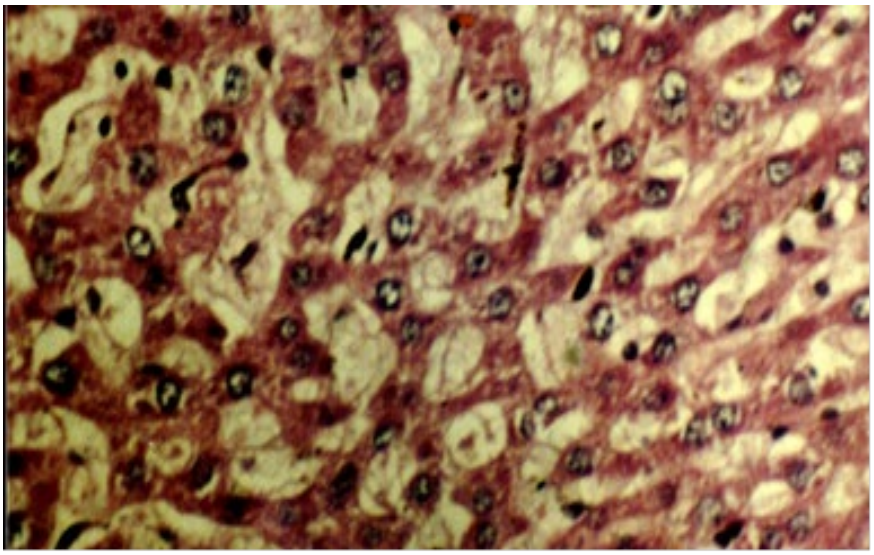

Figure 3.8b Microphotograph of Lung (treated) at HE × 100

3.23 Conclusion

The CMC used in the studies was produced to meet global pharmaceutical standards using good manufacturing practices (GMPs). Studies were taken into account in the safety assessment of CMC by the EU Scientific Committee for Food which allocated an ADI 'not specified' to this product in 1992 (SCF, 1992b). The present investigation demonstrated that the product had no statistically significant or treatment-related adverse effects on any of the parameters evaluated in safety trials. It is concluded that the results of the present study do not conflict with the general recognition of the human safety of CMC (SCF, 1992a; JECFA, 1990).

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CONCLUSION

The increasing environmental concerns have forced the researchers to obtain useful agrochemicals from agro biomass, a renewable resource that consists mostly of cellulose. From the analysis of composition of different parts of maize waste, it was found that the amount of cellulose was higher in husk waste than those of other parts of maize waste. Reduced cellulose particle size (i.e. 74 μm) was used as feedstock in carboxymethylation to get higher DS as DS is one of the main properties of CMC that decides its end use in industry. The highly water-soluble product obtained by the single step carboxymethylation reaction had higher DS 2.41, depending on the cellulose particle size as well as other reaction conditions. The highly purified food-grade CMC (99.95%) with higher yield (2.4 g/g) was successfully prepared that complied with the purity requirements of the EFSA, FCC, JECFA etc. The results of the toxicity study suggested that, the oral administration of CMC did not produce any significant toxic effect in mice and the NOAEL (no-observed-adverse-effect-level) was 20mg/g/day, highest dose tested. It is therefore justified to extend the recognition of safety granted to CMC. Hence, the obtained CMC product is well suited for pharmaceutical and food additives owing to its high purity. It is concluded that the production of food-grade CMC will not only be helpful in the urban agricultural waste management but also be fruitful in saving the country revenue which it expends in importing CMC.

LIST OF PUBLICATIONS BASED ON THE THESIS

LIST OF PAPERS

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LIST OF PATENT

1. Yeasmin *et al.,* Preparation of fluid disinfectant from castor oil. Patent application no. 86/2007, Serial No. 1004787**,** 2008**.**