CHANGES IN ENZYMATIC ACTIVITIES, BIOCHEMICAL PROPERTIES AND ANTIOXIDANT ACTIVITY DURING *KINEMA* FERMENTATION

by

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Changes in Enzymatic Activities, Biochemical Properties and Antioxidant Activity during *kinema* fermentation

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Approval Letter

The *dissertation* entitled "***Changes in Enzymatic Activities, Biochemical Properties and Antioxidant Activity during kinema fermentation***" presented by **Eljina Lawati Limbu** has been accepted as the partial fulfillment of the requirements for **Bachelors degree in Nutrition and Dietetics**

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Abstract

Kinema, a soybean fermented food is traditionally consumed by the Limbu and Rai, Nepalese inhabiting Nepal, India, some parts of Bhutan and Manmyar. This study was carried out with an aim to determine the changes in enzymatic activity, biochemical parameter and antioxidant activity during the 3 days *kinema* fermentation. For the *kinema* preparation, brown soybeans were cleaned, washed and soaked in water overnight at ambient temperature. During the fermentation process, the samples of every 12 h difference was collected for 3 days which was then taken for the study. Analytical procedures were done where enzymatic activity (Protease and amylase), physicochemical changes (Reducing sugar, Trichloro acetic acid soluble Nitrogen, protein, total phenolic content) and antioxidant activity were determined and compared between the samples. Similarly, pH values were also observed during each 12 hour fermentation time. The data were analyzed by using one way ANOVA using Genstat at 5% level of significance level.

The highest protease activity was found to be 0.190 ± 0.001 U/ml at 60 h of *kinema* fermentation which initially showed negative activity at 0 h (-0.008 U/ml). The pH value increased from 6.8 to 8.5 from 0 h to 72hrs of fermentation. Similarly, protein content was increased on the progressive interval of fermentation time with highest value of 2.758 ± 0.097 mg/ml at 72hrs of fermentation. Furthermore, Trichloro acetic acid soluble Nitrogen showed increasing trend till it reached 60 h of fermentation time with 101.382 ± 1.786 μg tyrosine equivalent/ml. Bioactive constituents like Antioxidant activity and Total phenolic activity was observed highest at 60h and 72h respectively. The higher level of the activity in *kinema* could be attributed to the extensive hydrolysis of proteins and an increase in the content of phytosterols. The study indicated that the Protease activity, protein, Trichloro acetic acid soluble Nitrogen along with release of bioactive constituents were higher during the third day of fermentation, while the amylase activity and the value of reduction sugar inclined during the first day of fermentation.

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List of Abbreviations

Abbreviation	Full form
ANOVA	Analysis of Variance
CCT	Central Campus of Technology
DFTQC	Department of Food Technology and Quality Control
DPPH	Diphenyl picryl hydrazyl
GAE	Gallic Acid Equivalent
WHO	World Health Organization
LSD	Least Significant Difference
MC	Moisture Content
TPC	Total Phenolic Content
RSA	Radical scavenging activity
MC	Moisture Content
IC50	Inhibitory concentration
d.f	Degree of Freedom
TCA	Trichloro acetic acid
Fig	Figure
SD	Standard deviation
DNSA	Dinitrosalicylic acid
Eqv	Equivalent

Part I

Introduction

1.1 General introduction

Ethnic fermented foods are produced, based on the indigenous knowledge of the ethnic people, from locally available raw materials of plant or animal sources either naturally or by adding starter culture containing functional microorganisms that modify the substrates biochemically and organoleptically into edible products that are culturally and socially acceptable to the consumers (Tamang, 2009). Due to the cultural and nutritional significance of traditional fermented foods, they are considered popular in Asia. Those fermented foods and beverages serve as vehicles for beneficial microorganisms that play an important role in human health and remain the oldest means of food processing and preservation (Anal, 2019). Primarily, fermentation was used to preserve foods, enhance shelf life, and improve flavor whereas in present context fermentation has been associated with many health benefits. During the process, microorganisms synthesize vitamins and minerals, produce biologically active peptides with enzyme such as proteinase and peptidase and also remove some non-nutrients (Sanlier *et al.*, 2017). Fermentation is a process that helps break down large organic molecules via the action of microorganisms into simpler ones. For example, yeast enzymes convert sugars and starches into alcohol, while proteins are converted to peptides/amino acids. The microbial or enzymatic actions on food ingredients tend to ferment food, leading to desirable biochemical changes responsible for the significant modification to the food. Fermentation is a natural way of improving vitamins, essential amino acids, anti- nutrients, proteins, food appearance, flavors and enhanced aroma. Fermentation also helps in the reduction of the energy needed for cooking as well as making a safer product (Sharma *et al.*, 2020).

The soybean or soy bean (*glycine max*) is a species of legume native to East Asia widely grown for its edible bean which has numerous uses like soy milk, soy sauce and fermented bean paste. Soybean is an important herbaceous annual plant in the family Fabaceae grown for its edible seeds. It is an important source of food, protein and oil and more research is essential to increase its yield under different conditions. The most important countries in the world with the highest rate of soybean production includes USA, Brazil, Argentina, China and India (Manandhar, 2021). Among cereal and other legume species, it has the highest protein content (around 40%); other legumes have a protein content between 20% and 30%, whereas cereals

have a protein content in the range of 8-15%. The soybean also contains about 20% oil, the second highest content among all food legumes(Liu, 2012). For more than thousand years, people in Asian countries have let the microorganism in the boiled soybeans increase. The traditional fermented products that use soybeans can be largely categorized into cases where fungi and Bacillus sp. are used. Likewise, Southeast Asian countries such as Korea, Japan and Indonesia have large amount of foods that uses fungi. Bacillus is widely used in fermented foods in many Asian countries such as India, Nepal, and Cambodia which produces viscous substances, which includes products such as Natto (Japan), Chongkukjang (Korea), kinema (India, Nepal, Bhutan), Thua nao (Thailand) (Shin and Jeong, 2015).

Kinema, a soybean fermented food is traditionally consumed by the non- Brahmin Nepalese inhabiting Darjeeling hills and Sikkim of India, Nepal and some parts of Bhutan. Kinema is a Nepali name which has very often been erroneously spelt as 'kenima'. *Kinema* production is a source of marginal income generation for many families in the Eastern Himalayas. Kinema is sold by rural women in all local periodical markets, locally called "*haats*", in eastern Nepal, Darjeeling hills, Sikkim, and southern parts of Bhutan. Usually, it is sold by volume taken in a small silver mug containing 150-200g of kinema, and packed in the leaves of a fig plant(*Ficus hookeriana*) locally called "nevara", then tied loosely by straw (Tamang, 2015). Likewise, the preparation of *kinema* is very similar to *natto*. However some of the steps in kinema preparation are different from the preparation of natto which makes kinema a unique non salted soybean fermented product (Sarkar *et al.*, 1994). Nowadays, *Natto* is produced by a controlled fermentation process using a pure culture starter, while kinema is allowed to undergo natural fermentation without adding a starter. The flavor of *Natto* is produced during the fermentation of soybeans with *B.subtilis* (natto), and the taste comes mainly from the hydrolysate of the soybean proteins (Nikkuni *et al.*, 1995). Soybean and different soybean products are known to contain phenolic compounds. Concentrations of these compounds in soybean were reported to increase after fermentation. It was suggested that the increased antioxidant activity was due to the liberation of lipophilic aglycones of isoflavone glucosides (Moktan *et al.*, 2008a).

1.2 Statement of the problem

More than 90% of the people of Nepal are involved in the agricultural profession. More of the traditional technologies are passed on as trade secrets in families of certain communities, a practice protected by tradition. Traditional foods, both fermented and nonfermented, have been the basis and are equally important for food security, preservation, and cultural and ethical

practices. However, complete scientific information on these various food products, their traditional ethics, production and preparation methods, and mode of consumption of these products are lacking (Dahal *et al.*, 2005). Although there are numerous significances regarding the cultural and nutritional aspect on fermented food *kinema*, there are people who are reluctant to include *kinema* on their diet. There are several of scientific research conducted on other soybean fermented product yet kinema has remained in obscurity. Despite the fact that large quantity of soybean is grown in Asia and can play major source to reduce malnutrition, the use of such beneficial soybean product is uncommon due to the lack of proper findings and research. The product has not received considerable attention although they are claimed to reduce the formation and progression of certain types of cancers and some chronic diseases such as cardiovascular disease, Alzheimer's disease and osteoporosis. Regarding *kinema*, there has been studies for its changes in microflora but there are few studies on bioactive principles and enzymatic activities. Although higher phenolic and antioxidant activities were found in *kinema*, as compared to the unfermented soybean seeds. However, the studies on changes in these characteristics with fermentation time are somewhat still lacking (Katuwal *et al.*, 2023).

Despite the availability of *kinema* in the market, the preparation methods are not standardized. These commercially available products are not consistent with their nutritional quality, production methods and organoleptic quality. Similarly, there are insufficient regulations governing the production and labeling of *kinema*, allowing for a wide range of quality and standards. Fermentation methods may vary significantly between producers, impacting the microbial strains used, fermentation time, environmental conditions, leading to product inconsistency.

1.3 Objective of the study

1.3.1 General objectives of the study

The general objective of this study is to determine the changes in Biochemical parameter, enzymatic activity and antioxidant properties during kinema fermentation prepared from brown soybean.

1.3.2 Specific objectives

The specific objective of the study will be

i. To prepare *kinema* in laboratory.

ii. To study the enzymatic activity (protease and amylase) during *kinema* fermentation.
iii. To study the changes in antioxidant activity and total phenolic content during *kinema* fermentation.
iv. To evaluate physicochemical changes during *kinema* fermentation.

1.4 Significance of the study

From traditional point of view, preservation of indigenous foods plays important role in the reflection of tradition and heritage of a whole nation. *Kinema* being an indigenous non-salted fermented soybean food commonly consumed in eastern hilly region of Nepal and in North Eastern Hills of India bordering Nepal as a seasoning agent plays significant role in promotion of traditional foods. Such traditional fermented foods have a tremendous potential for alleviating protein energy malnutrition, as they are nutritious, cheap and easy to prepare. Similarly, from nutritional point of view, in comparison to soybean, *kinema* proteins are good source of almost all EAAs (Essential Amino Acids) and that their score is as high as that off egg and milk proteins.

Although *kinema* is a common dietary component in many inhabitant of hilly region, the influence of fermentation on the nutritional aspect is yet to be fully determined. This study is expected to provide a wider perspective on changes that occur during fermentation process, which can have potential benefits. By observing the fluctuations in certain intervals of time, it aims to standardize the procedure and give closer view on the kinema fermentation. Furthermore, this study also aims to determine the most suitable time frame for *Kinema* fermentation to prepare best *kinema* in terms of nutrition and its functional properties . Beside this, the study highlights the importance of *kinema* with respect to antioxidant activities and release of polyphenolics, active peptide fermentation during *kinema* fermentation, and these information increase the importance of kinema as a functional food. Similarly, the study thus helps to grow food industry and draw proper attention towards reforming its image as a therapeutic food.

1.5 Limitation of the study

1. The microbial analysis could not be done.
2. Mineral and amino acid contents were not analyzed

Part II

Literature review

2.1 The origin and distribution of soybeans

It is widely believed that the soybean originated in China, probably in the north and central regions, 4000-5000 years ago. The first written record of the plant is contained in the book Materia Medica by Chinese Emperor Shen Nong in about 2838 B.C. describing the plants of China. The soybean, then known as *shu,* is repeatedly mentioned in later records and was considered one of the five sacred grains, (or *wu gu* in Chinese) along with rice, wheat, barley and millet which were essential to Chinese civilization (Liu, 2012). As with most important food plants, the early history of the soybean is lost in obscurity. According to Piper & Morse it was undoubtedly utilized, if not cultivated, by primitive man long before the dawn of historical records. The first known records, however, indicate that soybean emerged as a domesticated crop around the eleventh century BC in China. Production was largely confined to the orient until after the first three decades of the twentieth century. In the last 1940s, however, the United States overtook China and soon the entire orient in soybean production (Turnipseed and Kogan, 1976).

Soybean (Glycine max) is a leguminous crop called as kind of legume, the “miracle crop”, and “gold from the soil” and in Nepali language, it is called Bhatmas. The most dominant varieties, color of seeds and locations, it has been given different local name that includes Nepale, Hardi, Saathiya, Darmali, Maily, Kalo and Seto (Pradhananga, 2019). Soybean, a self-pollinated crop is one of the most important oil and protein crops of the world. The soybean is an excellent source of major nutrients including a good source of vitamins and minerals. Instead of producing oil, the seeds of soybean could also be used for producing many food dishes, confectioneries, baby foods, etc. The protein of soybean is called a complete protein, because it supplies adequate amount of different amino acids required for building and repairing the damaged body tissues (Islam *et al.,* 2007).

The world soybean production in 2017 was 352.6 million tons (Sobko *et al.,* 2020). The role of soybean isoflavones in the prevention and treatment of several diseases were presented, which comprise antitumor, antimenopausal (female) osteoporosis and antiaging properties improvement of learning and memory skills of menopausal women, prevention and treatment of heart disease diabetes and kidney disease (Wang *et al.*, 2013).

2.2 Botanical profile

Soybean plant is an erect, bushy and leafy summer annual herb that reaches a height of 25 to 50 inches. It has alternate, trifoliate leaves except the leaves of first two cotyledonary nodes. Its leaves, stems and pods are covered with grey or brown hairs. Flowers are borne in the auxiliary position and are usually either purple or white. Soybeans grow best in areas having hot, damp summer weather but they can be grown under a great variety of climatic conditions. The growing seasons for soybean varieties is controlled by their response to photo period and temperature. The number of days from planting to maturity can range from 80 to 180 days, depending on the varieties and environmental conditions. The Taxonomical hierarchy of Soyabean is given below:

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Subfamily: Faboideae

Genus: *Glycine*

Species: G. max

Botanical name: Glycine max (L.) Merr.

Common name: Soybean, Soya bean

(Badole and Bodhankar, 2012)

2.3 Chemical and nutritional composition of soybeans

On average, soybean seeds contain 18-25% oil and the by-product of oil extraction is soybean meal, containing 40-44% crude protein. The concentrations of these two components are genetically predetermined but can vary depending on the environment. The protein and oil content of soybean seeds are negatively correlated. A higher temperature during reproductive growth tends to result in higher oil content (Sobko *et al.*, 2020). The major fraction of soy protein is termed globulin (a salt soluble fraction) majority of which can be extracted with water. These are relatively insoluble in pH close to their isoelectric pH(4.5), their solubilities being highest between 2.0 to 3.5 and pH >7.0. Being composed of both polar and non-polar amino acids, soy proteins can bind to water and to lipids and thus acts as protective colloids and emulsion stabilizers (Snyder and Kwon, 1987). Soya bean (Glycine max) proteins are well balanced in the essential amino acids, except slightly limiting in methionine. Whole soya bean flour is a good source of dietary fibers, it is very rich in hemicellulose and cellulose and lignin levels are low. Consumption of moderate amounts of soya bean seed has been shown to produce beneficial effects on glucose and lipid metabolism in man (Bau *et al.*, 1997). Soyabean is of particular interest as a vegetable protein source because of its cholesterol-lowering abilities in patients with type II hyperlipoproteinemia. Soyabean is also rich in minerals and vitamins such as iron, Zinc, copper, thiamine, riboflavin, niacin and pantothenic acid (Etiosa *et al.*, 2017). The proximate composition of soybean is given in table no. 2.1.

2.4 Anti-nutritional factor of soybean

Amounts of dietary essential amino acids (EAA), digestibility of proteins, and bioavailability of amino acids are basic are basic parameters in determining the quality of a protein source. A number of anti-nutritional factors are present in food and feed products that may adversely affect protein digestibility and amino acid availability. Antinutritional factors may occur naturally, for instance, glucosinolates in mustard and rapeseed protein products, trypsin inhibitors and hemagglutinins in legumes, tannins in legumes and cereals, phytates in cereals and oilseeds (Gilani *et al.*, 2005). The presence of anti-nutritional impairs the nutritional quality of legumes such as significant amount of trypsin inhibitor. It has been reported that upon feeding they depress animal growth, decrease dietary protein digestibility, reduce the Sulphur and nitrogen absorption, stimulate pancreatic enzyme secretion, induce pancreatic enlargement and increase the synthesis and release of hormonal factors. Soytoxin induces tonic-clonic

convulsions and flaccid paralysis followed by convulsions and death (Vasconcelos *et al.*, 1997).

Table 2.1 Proximate composition of soybean (DFTQC, 2017)

Parameter	Black Soybean	Brown Soybean	White Soybean
Moisture(g)	12.1	8.1	10.2
Protein (g)	33.3	43.2	33.3
Fat (g)	15	19.5	17.7
Carbohydrate(g)	31.3	20.9	29.6
Minerals (g)	4	4.6	5
Fiber (g)	4.3	3.7	4.2
Calcium (mg)	213	240	226
Phosphorus (mg)	509	690	546
Iron(mg)	9.5	10.4	8.5
Carotene(µg)	10	426	10
Thiamine (mg)	6.65	0.73	0.66
Riboflavin (mg)	0.23	0.39	0.22
Niacin (mg)	2.8	3.2	2.2
Energy (kcal)	393	432	411

2.4.1 α-Galactoside carbohydrates

On maturation, soy beans contain about 12-12.5% soluble total sugar of which the raffinose sugars comprise approximately 5.3-5.6% of the dry weight and are an important energy source for seeds during germination and early growth of plant. Sucrose, raffinose and stachyose are the principal oligosaccharides of this group. The principal α-galactosides of soya beans are raffinose and stachyose, characterized by α-galactoside bonds which cannot be hydrolysed due to the absence of α-galactosidase in the human intestinal mucosa (Bau *et al.*, 1997).

2.4.2 Lectins

Lectins are proteins with the property of agglutinating the erythrocytes of higher animals. Lectins are one of the most important toxic constituents of pulses. They cause growth retardation and in extreme cases few ending in fatality of the animals. It was reported that dietary soya bean lectin can inhibit growth and induce considerable changes in the physiology of the pancreas and small intestine and may therefore, in part, contribute to the poor performance observed in animals fed on soya beans (Bau *et al.*, 1997).

2.4.3 Trypsin Inhibitors

Trypsin inhibitor (TI) has been implied to be one of the factors responsible for reducing protein digestibility, pancreatic hypertrophy, and poor growth performance in rats, mice, and chicks (Sarkar and Nout, 2014).

2.4.4 Phytic acid

Phytic acid, the hexaphosphates ester of myoinositol, is a major phosphorus storage constitute of most cereals, legumes and oilseeds. The ability of phytate to complex with proteins and with minerals and the consequences lead to decreased protein and mineral solubility and hence affect protein properties and mineral bioavailability. They are widely distributed in mature legume grains, stores most of the grain phosphorus. Phytic acid chelates several minerals and thereby reduces their bioavailability (Nolan *et al.*, 1987).

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) is widespread in whole grain and oilseed food products: 1 g of soya bean flour contains approximately 4 mg phytate, representing 57% of the total organic phosphorus. Phytates play an important role in mineral availability. Phytic acid decreases the availability of zinc, magnesium, iron as well as

protein. When bound to protein, it induces a decrease of solubility and functionality of the protein (Bau *et al.*, 1997).

2.5 Methods to reduce Antinutritional factors

The antinutrients in plants reduce the absorption of nutrients from the digestive system and are of particular concern in societies that base their diets largely on grains and legumes (Savage and Klunklin, 2018). Simple ways to reduce the amount of antinutrients in foods are soaking, Germination, Boiling, Roasting and Malting. Since many antinutrients are water-soluble, they simply dissolve when foods are soaked. In legumes, soaking has been found to decrease phytate, protease inhibitors, lectins, tannins and calcium oxalate (Bishnoi *et al.*, 1994). Sprouting is a period in the life cycle of plants when they start emerging from the seed. This natural process is also known as germination.

During sprouting, changes take place within the seed that lead to the degradation of antinutrients such as phytate and protease inhibitors. This process increases the availability of nutrients in seeds, grains and legumes (Bau *et al.*, 1997). Similarly, in various grains and legumes, fermentation effectively degrades phytate and lectins. For example, fermenting pre-soaked brown beans for 48 hours caused an 88% reduction in phytate (Gustafsson and Sandberg, 1995). High heat, especially when boiling, can degrade antinutrients like lectins, tannins and protease inhibitors (Egbe and Akinyele, 1990). The cooking time required depends on the type of antinutrient, food plant and the cooking method. Generally, a longer cooking time results in greater reductions of antinutrients. On the other hand, roasting can improve protein digestibility. Roasting reduces the amount of aflatoxins produced by fungi (Samarajeewa *et al.*, 1990).

2.6 Soybean products

Soyfoods are commonly divided into two groups mainly non-fermented soyfoods and fermented soyfoods. Fresh soybeans, dehydrated soybeans, soy sprouts, whole-fat and defatted soybeans, soy flour, soymilk and its products, tofu belongs to non-fermented soyfoods whereas fermented soyfoods include miso, soy sauces, natto, tempeh, and fermented tofu (Chen *et al.*, 2012). In South-East Asia, the preparation and consumption of fermented soybean foods is a traditional art of the people. Similar to *Chungkokjang*; a fermented soybean paste consumed as soup with boiled rice in Korea, *Natto*; a highly sticky fermented soybean food consumed in Japan, *Kinema* is a whole soybean non salty fermented food with sticky texture, gray tan in

colour and a characteristic flavor eaten as side-dish in the Eastern Himalayan regions of Nepal, the Darjeeling hills and Sikkim in India and in Bhutan (Tamang, 2002). *Natto* is a traditional fermented soybean food product with venerable history in Japan. By steaming soybeans and inoculating with *Bacillus subtilis*, natto spores to ferment it, and then it would become a characteristic odor, flavor and slimy food (Weng and Chen, 2010a). Similarly, such foods including 'thau-nao' in Thailand, 'tao-si' in the Philippines shows that fermentation is widely used in the food industry not only to improve the sensory characteristics of a product, but also to eliminate certain undesirable constituents, make nutrients more accessible while preserving and even improve the nutritional properties (Hu *et al.*, 2010). Some popular traditional fermented soyfoods and their sources other than kinema are listed in table 2.2.

2.6.1 Kinema

Kinema is a unique flavorsome delicacy to many people of Nepal and the neighboring countries. This indigenous soybean-fermented condiment is a low-cost meat substitute and a source of income for many rural households (Sarkar and Nout, 2014). It is solid-state alkaline fermented soybean food product of the eastern hills of Nepal which is mainly consumed by non-Brahmin Nepalese inhabiting Nepal, Darjeeling and Sikkim of India. It is known to have a pungent smell of ammonia, slimy texture and short shelf life. *Kinema* after fermentation possess stringy threads when touched with fingers and it is believed that the longer the threads, the better the quality of *kinema* (Khadka and Lama, 2020). This traditionally prepared, non-salted fermented product of soybean that contains abundant amount of phenolics which are converted to a more bioactive and bioavailable form of fermentation. Moreover, this fermented soybean product contain peptides having antioxidant, antitumor, antidiabetic, antimicrobial and Angiotensin I converting enzyme(ACE) inhibitory properties (Katuwal *et al.*, 2023).

2.6.2 Origin and culture

Traditionally fermented foods are mostly specific to certain geographical regions and also particular communities. These are manufactured in bulk quantities and consumed every day possibly due to poor shelf life. These foods are the important part of the dietary system of all sections of the people, irrespective of economic disparities. Such products are mainly prepared from the local abundantly available agricultural commodities in that particular region (Shrestha *et al.*, 2002).The word "kinema" is derived from "kinambaa" of the Yakthung or Limbu (a tribe indigenous to Limbuwan comprising nine districts within Mechi and Koshi zones of east

Table 2.2 Some popular traditional fermented soyfoods and kinema :(Chen *et.al*, 2012)

Products	**Description**	**Origin**
Chungkookjang/ Cheonggukjang	Steamed black soybean fermented with *B. subtilis* for several days without salt	Korea
Doenjang	A traditional Korean condiment made from soybean paste block and rice by *B. subtilis*	Korea
Miso	A traditional Japanese seasoning produced by fermenting rice and soybeans with salt and the fungus kojikin	Japan
Natto	A traditional Japanese soybean product fermented with *B.subtilis*	Japan
Tauco	A Chinese-Indonesian cuisine paste made from preserved fermented soybeans	Indonesia
Tempeh	A traditional fermented soybean cake from black or yellow soybean fermented with *R. oligosporus*	Indonesia
Thua-nao	A traditional non-salted whole soybean fermented like Japanese natto by mixing with *Bacillus* spp.	Thailand
Kinema	A nonsalted and solid-state alkaline fermented soybean food product of the eastern hills of Nepal. It is mainly consumed by non-Brahmin Nepalese inhabiting Nepal, Darjeeling and Sikkim of India, and in some parts of Bhutan.	Nepal

(Khadka and Lama, 2020)

Nepal) dialect where “ki” means fermented and “nambaa” means flavor. Due to mixed society of multiethnic communities this might have resulted in the spread of kinema culture to the allied Nepali communities, such as the Rai, Tamang, Gurung, and Mangar. Because of the trans-boundary movement of the people, kinema is also popular in the adjacent Darjeeling district of the State of West Bengal and the State of Sikkim in India, and also in Bhutan (Sarkar and Nout, 2014). *Kinema* is an ethnic fermented soybean food of the Nepali community in the Eastern Himalayas made of whole-soybean ; it is a sticky, slightly alkaline product with a slight ammonical flavor that is produced by natural fermentation, which is gray tan color and is flavorsome. Likewise, It is interesting to note that mountain women use their indigenous knowledge of food production to prepare kinema. This unique knowledge of kinema-making has been protected as an hereditary right and passed from mother to daughter, mostly among the Limboo (Tamang, 2015).

2.6.3 Methods of preparation

In the traditional method of preparation, soybeans are cleaned, washed, soaked in water overnight at ambient temperature (10-20°C) And then the excess water is drained off. After that the soaked soybeans are cooked until they can be crushed easily between the fingertips. Then the water is removed and they are crushed lightly by a wooden pestle to dehull the seed. This practice of cracking the cooked seeds of soybeans is observed only during kinema production, unlike other similar fermented soybean foods of Asia and north-east India, probably to increase the surface area for seed fermentation by aerobic spore-forming Bacillus species (Tamang, 2015).

The soybean grits containing torn hulls are then wrapped with fresh fern (*Athyrium* sp.) or *Leucosceptrum canum* smith leaves covered with sackcloth and kept in a bamboo basket above an earthen stove in the kitchen to ferment for 1-3 days. The desired state of fermentation is determined primarily by a typical kinema flavor dominated by ammonia as well as the development of a rough, white viscous fluid on the beans. This fluid has the property of forming long stringy threads when touched and stretched with thumb and middle finger; the better is the quality of kinema can also be determined by presence of long stringy threads.

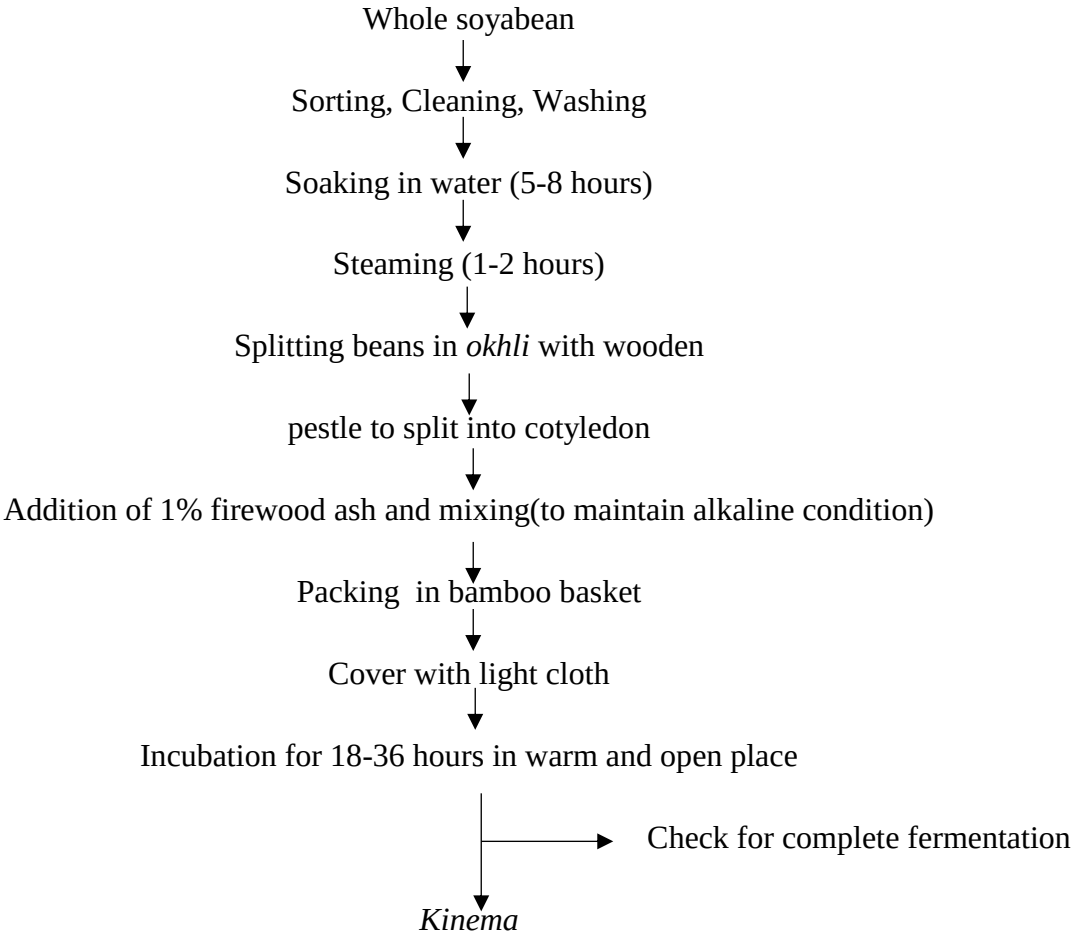
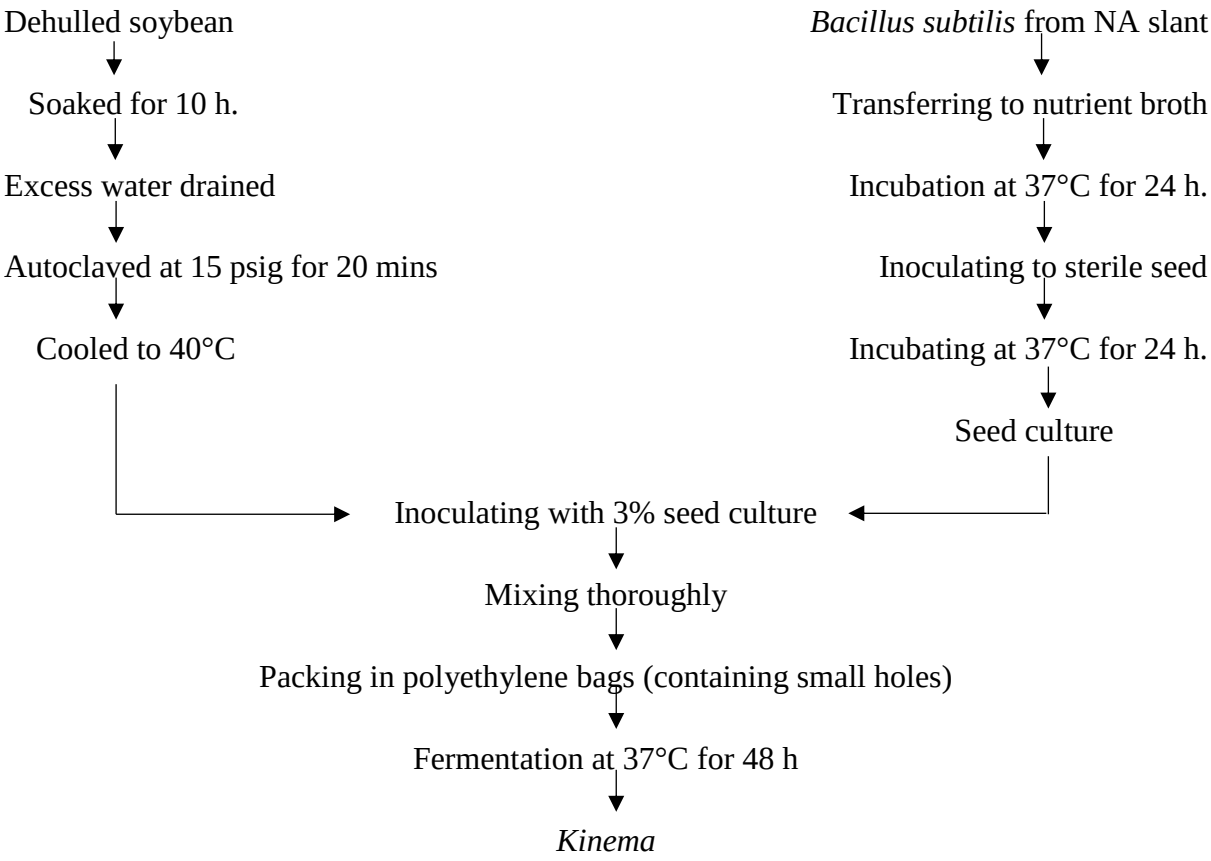


Fig. 2.1 Flow sheet of kinema preparation by traditional method

(Shrestha, 1989)

Hence the combination of a mild smell of ammonia, a grayish brown color, and is semi-hard, like raisins accompanied by a nutty flavor gives good quality kinema (Dahal *et al.*, 2005).



(Nepali, 2007)

Fig. 2.2 Flow sheet of *kinema* preparation by pure culture method

2.6.4 Proximate composition of *kinema*

The moisture content of kinema was 62%; on dry basis, *kinema* contained about 48% protein, 17% fat, 28% carbohydrate and 7% ash and the energy value was 2.1 MJ/100g. Similarly, the moisture content, protein, fat, carbohydrate, ash and the energy value of soybeans were 11%, 47%, 22%, 26%,5% and 2.1 MJ/100 g respectively. While the pH of raw soybeans was neutral to slightly acidic (average 6.75), that of kinema was distinctly alkaline (average 7.89). Free fatty acidity in kinema was about 33 times higher than raw soybean (Sarkar *et al.*, 1994). The proximate composition of soybean and kinema is presented in table 2.3.

Table 2.3 Proximate composition of soybean and kinema

Parameters	**Soybean (db)**	**Kinema (db)**
Moisture content (%)	11	62
Protein (%)	47	48
Fat (%)	22	17
Carbohydrates (%)	26	28
Ash (%)	5	7
Energy value (MJ/100g)	2.1	2.1
pH	6.75	7.89

(Sarkar *et al.*, 1994)

2.6.5 Micro-flora in Kinema

The dynamics of microbial succession in fermented food is a complex phenomenon. The microbial flora and the physical and chemical characteristics of the matrix influences the behavior and survival of some of the microorganisms, while death and disappearance of the others (Shrestha *et al.*, 2002). Likewise, The fermented soybeans (FSB) of three Lanna ethnic groups in Northern Thailand-*Karen*, *Lawa* and *Shan* contained *Bacillus* as a major bacterial group. The profile of minor bacteria was unique for the FSB of each ethnic group, which could be attributable to the geographical environment and the FSB production processes. Some of the minor bacteria, considering their natural habitats, could be linked to inadequate hygiene practices during food production. Some members of the *Bacillus* genus in all FSB_s as well as *Vagococcus* in the *Shan* FSB could potentially be probiotics or beneficial to human health (Yongsawas *et al.*, 2023).

Several species of *Bacillus* have been isolated from *kinema* including *Bacillus subtills, Bacillus licheniformis*, *Bacillus cereus*, *Bacillus circulans*, *Bacillus thuringiensis* and *Bacillus sphaericus* at an average load of 10^8 colony forming units (CFU)/g. The equipment and leaves used as wrapping materials harness microbiota for natural fermentation of *kinema.* Likewise, the freshly prepared kinema if kept below 10°C for 1 day stabilizes the quality of the product by preventing further biological activity of microorganisms and show better stickiness, which is a very important sensory property of *kinema* (Tamang, 2015). *Bacillus subtilis*, *Enterococcus faecium* are the major microflora reported to be associated in kinema fermentation as bacteria whereas *Candida paraplosis* and *Geotrichum candidum* as yeast. *Bacillus subtilis* being the most dominant organism during the kinema fermentation, the completion of fermentation is generally indicated by appearance of white viscous mass on the soybean seeds and typical kinema flavors with a slight odour of ammonia (Tamang, 2009).

The high prevalence of *B. subtilis* and *Enterococcus faecium* in *kinema* samples indicate their possible involvement in the production of *kinema.* Furthermore, it has been reported that in several soybean-based foods, such as Natto and thua-nao, and the African locust bean food, daddawa, the involvement of *B. subtilis* was reported. The presence of *E. faecium* has been reported in gari, a popular cassava tuber fermented food of West Africa. This species is present in several nonstarch foods, but the source is not from faecal contamination. However, the *Bacillus* fermentation of soybeans to produce kinema was quite unaffected by the presence of *E. faecium* in terms of proteolytic activity, ammonia production and final pH of the fermentations. Although E. *faecium* does not add any sensory quality to the Bacillus fermentation of soybeans, it is always encountered in naturally fermented *kinema*. Presence and growth of yeast during kinema preparation are associated with the development of rancidity in the products (Tamang, 2015). The lower frequency of Candida parapsilosis and Geotrichum candidum in kinema indicates that they may be opportunistic organisms, having no involvement in fermentation. The aerial contamination by yeasts in foods has been reported by Sandhu and Waraich (1981). Candida parapsilosis has been found in ragi and *G. candidum* has been reported in many fermented foods (Sarkar *et al.*, 1994).

2.7 Changes in the Substrate during Kinema production

A number of interesting changes occur during processing of soybeans to produce *kinema*. During soaking of soybeans, there is no significant change in the frequency of coliforms and *Bacillus subtilis* cells present on raw soybeans, but a population of lactic acid bacteria appears,

resulting in a decline in pH of soaked beans from 6.6 to 5.3 (Sarkar, 2000). Similarly, the subsequent process of steaming kills most of the bacteria in soaked beans leaving a near pure culture of *Bacillus,* which survives as spores and can grow without competition (Nout and Rombouts, 1990).

2.7.1 Changes in Proximate composition during kinema fermentation

During the fermentation of *kinema,* it was found that before 16 h of fermentation, there was no significant increase in the contents of Free Fatty Acids (FFA) and non-protein nitrogen, suggesting that sugars, not fats or proteins, are initially used as substrates for metabolism and growth of *B.subtilis.* Likewise, It seems that a rise in protein content is related to an increase in microbial synthesis of protein or enzymes or chemical rearrangements followed by degradation of the other compounds. The effect of fermentation on ash content is inconclusive: a decrease from 4.8% to 2.3% and an increase from 5.0 to 5.6%-7.4%. Carbohydrates have been reported to decrease owing to bacteria using them for energy supply. *Kinema* fermentation decreases total sugar levels in soybeans while increasing reducing sugar levels. Soybean crude fiber levels are reduced throughout fermentation as a result of hydrolysis by carbohydrate-splitting enzymes from *B. subtilis*. The organism was obligately aerobic and hydrolyzed gelatin and tributyrin (Sarkar and Nout, 2014).

2.7.2 Changes in Amino acids during kinema fermentation

The very high acidic amino acid content of *kinema* is of particular interest. Glutamic acid is the most abundant amino acid, followed by aspartic acid, together representing 31% of total residues (Sarkar *et al.,* 1997). The strong proteolytic activity results in a significant decline in protein nitrogen content, with a concomitant increase in non-protein and soluble nitrogen contents. Likewise, the free amino acid content of unfermented soybeans, which is only 0.18% of the total dry mass, increases 60-fold in kinema. The percentage liberation of amino acids (based on the ratio of sum of the free amino acid contents to that of the total amino acid contents) at the time of fermentation was 37 (Sarkar and Nout, 2014).

In case of traditionally prepared *kinema* the percent liberation of amino acids was 11.4 only (Nikkuni *et al.,* 1995). The net decrease in some individual amino acid levels after fermentation suggests that they are metabolized to a greater extent than they are replaced by proteolytic activity. The decrease is particularly pronounced for arginine and cysteine. In addition, levels of some amino acids (alanine, isoleucine, serine, aspartic acid, asparagine, arginine, tyrosine,

methionine, and hydroxyproline) are significantly depleted when yeasts contribute to the B*acillus* fermentation of soybeans. It is also observed that *kinema* proteins are a good source of almost all the essential amino acids (Sarkar *et al.*, 1997).

2.7.3 Changes in lipids during kinema fermentation

It was observed that there was increase (20-30%) in crude lipid content of soybeans after fermentation. this is probably due to active assimilation of carbohydrates and limited consumption of lipids, resulting in an enrichment of crude lipid at the end of fermentation (Sarkar *et al.*,1996). The significant increase in FFA is 16%-27% during fermentation and hence the FFA content is 3.1%-3.7% of crude lipid. The increased fatty acid levels in fermentation products may be due to the synthesis and accumulation of extra lipids or hydrolysis of glycerides during fermentation. Another possibility could be the dissociation of lipoprotein complexes in soybeans during fermentation, resulting in the release of ether-extractable FFAs (Wang *et al.*,1975).

2.7.4 Changes in Vitamins during kinema fermentation

Bacillus fermentation enhances thiamine, riboflavin, and niacin levels by 45, 71 and 23 % respectively. However, the levels decline by 31, 18 and 74 %, respectively, in the presence of *E. faecium*, indicating that this bacterium uses readily available vitamins for growth and metabolism. In terms of overall B-group vitamin content, *Bacillus*-fermented *kinema* produced at 37°C for 48 h is superior to that prepared by other fermentation conditions. Increase in these vitamins as a result of fermentation has important nutritional implications in communities where this food contributes to the formation of the only side dish of rice (Sarkar and Nout, 2014).

2.7.5 Changes in Minerals during kinema fermentation

Kinema contains significantly lower levels of minerals than found in raw beans as the production process involved soaking as well as cooking. Despite such a large loss in minerals during processing, *kinema* (prepared in laboratory using distilled water for soaking and cooking and without adding firewood ash) still contains appreciable quantities (4, 10, 13, 18, 683, 494, 1257, and 2077 mg/kg dry weight) of copper, manganese, Zinc, iron, calcium, magnesium, Phosphorus, and Potassium, respectively. *Kinema* is not only a valuable source of minerals

particularly where intake from other sources is marginal but also an important potassium-rich diet (Sarkar *et al.*, 1998).

2.7.6 Reduction of Anti-Nutritional factors during kinema fermentation

During *Kinema* production, after soaking and cooking there was 44.9% reduction in the phytic acid content of raw soybeans (8.44 g/kg dry weight). Likewise, monoculture fermentation caused a further reduction of the content in cooked beans by 58.4%. It was observed that Trypsin inhibitor activity reduced by 43.5% after soaking and cooking, and 66.2% after monoculture fermentation. Furthermore, only 5% of the hemagglutination activity remained after soaking and cooking soybeans which was fully destroyed after fermentation during the production of *kinema* (Sarkar and Nout, 2014).

2.8 Enzymes involved in kinema

Soybean is known to be rich in proteins and several other bioactive components including phenolic acids, isoflavones and flavanols, which can be converted to more bioavailable and bioactive form during fermentation (Cho *et al.*, 2011). Proteases, commonly known as proteolytic enzymes or proteinases, are hydrolytic enzymes. They work by hydrolyzing peptide bonds and converting proteins into shorter peptides for many purposes. Proteases are one of the most important classes of proteolytic enzymes widely distributed in the animal kingdom, plant as well as in microbes. They are found in many types of microorganisms, including bacteria, actinomycetes, viruses and fungi. *B. subtilis* is regarded as key organism responsible for fermentation of different varieties of alkaline traditionally fermented soybean products. According to (Sarkar and Tamang, 1995a), protease from *B. subtilis* degrades soy proteins, resulting in a considerable increase in non-protein and soluble nitrogen, but a decrease in protein nitrogen, for example, from 7.7% N (47.8% protein) to 6.3% N(39.4%).

Due to the production of specific hydrolytic enzymes such as proteases, α-amylase, cellulose, pectinase and β-glucosidase, *Bacillus* spp. have been used for solid state fermentation of plant matrix including agricultural by-products (Rai *et al.*, 2017). Several proteolytic enzymes have been shown to serve regulatory roles in a variety of physiological functions. Certain regulatory proteases, such as fibrinolytic and caseinolytic, were shown to be more active in *natto* made from black soybeans (than soaked beans). These enzymes can dissolve fibrin clots, which can be employed to treat damaged vascular systems. *B. natto* fermentation in soybeans is capable of producing (or enhancing) a-glucosidase in soybeans, which may

accelerate breakage of a-glucosyl bonds in black soybean glucoside isoflavones to create aglycones. Furthermore, the fermentation of black soybeans with the tested starter microbes resulted in a significant increase in aglycone and total anthocyanin content.

These enzymes possess the huge commercial potential and thus have been used in several industrial processes, including food industry, leather processing, silk processing, detergent industry and therapeutic applications (Srilakshmi *et al.*, 2015). Plant parts including flowers, seeds, roots and leaves can be aqueously macerated to extract plant proteases from natural sources. Since degree of purification, the crude extract thus obtained may be further processed to obtain either a partly purified enzyme or a pure enzyme. Plants require proteases for a variety of physiological and developmental activities from seed germination to plant death. Since protease is present in every plant tissue, it may be isolated from natural sources or synthesized in vitro (Gonzalez-Rabade *et al.*, 2011).

Amylases are digestive enzymes which hydrolyze glycosidic bonds in starch to glucose, maltose, maltotriose and dextrin. Although amylases can be derived from several sources, such as plants, animals and microorganisms, the enzymes from microbial source generally meet industrial demands and had made significant contribution to the production of foods and beverages (Singh *et al.*, 2011). 0-8% Amylase can be produced by either submerged fermentation or solid-state fermentation, both methods defend on the physiochemical factors or parameters (Ahmad *et al.*, 2019).

2.9 Enzymatic changes during kinema fermentation

The kinema organism *B. subtilis* produces strong proteolytic enzymes which hydrolyze the protein into peptides, amino acids, ammonia and other flavoring compounds. Proteolysis increases the solubility of protein and improves other functional properties as well (Sarkar and Nout, 2014). The major metabolic activity of the bacteria is proteolysis of the legume proteins and utilization of the released amino acids. As a consequence ammonia is formed and the pH value rises. When the pH value reaches about 8-8.3, sufficient ammonia (pK_a 9.2) is present as volatile NH_3, for the product to have an unpleasantly strong ammonia odour which readily reaches objectionable levels (Allagheny *et al.*, 1996).

During fermentation, it was observed that one of the most remarkable features of the genus *Bacillus* is the secretion of various extracellular enzymes, including protease, amylase, γ-glutamyl transpeptidase (GTP), levansucrase, and phytase. As natto bacilli grow, the enzymes

that they secrete catalyze many reactions that lead to production of the characteristic sticky material as well as formation of the characteristic aroma and flavor (Liu, 2008). *B.subtilis* exhibits significant proteolytic activity as evidenced by the gradual rise of trichloroacetic acid-nitrogen (TCN-N) and ammonia nitrogen with *natto* fermentation, which almost multiplied after 48 h of incubation. TCA-N increased continuously with the time course of fermentation and generally preceded the observed change in ammonia nitrogen. A similar observation was made during a study on '*thua-nao'*, a traditional Thai-fermented soy product where proteinases released by the dominant species in the inoculum, especially *B. subtilis*, play significant role in proteolysis of soy proteins during fermentation (Sarkar and Nout, 2014).

During tempeh fermentation, as microorganisms grow, they produce various enzymes, which breakdown soybean components. This leads to composition changes. Compared with miso and soy sauce, these changes are much less vigorous due to limited production of enzymes by the tempeh mold. Similarly, during koji (intermediate product for making various fermented food products such as fermented soy paste and soy sauce) making, various enzymes are produced which includes protease, amylase, glutaminase, lipase, hemicellulose, pectinase, and esterase. These enzymes become active during fermentation which results in the degradation of the original material (Liu, 2008).

Amylase activities in wheat and rye sourdough liberate maltodextrins, maltose, and glucose during fermentation. In simulated sourdough fermentations without microbial activity, maltose accumulates at the initial stage of fermentation. After reduction of the pH of 4.5, maltogenic amylases are inhibited but glucoamylase continues to release glucose from starch and maltodextrins (Gänzle, 2014). Lipase activity was detected late compared to the other enzymes studied. Considering the fact that soybean is an oil-rich seed, the low lipase activity was rather surprising was rather surprising but not unique since similar observations have been reported in the fermentation of the oil-rich seeds. Thus, the activity of lipase has never been considered to be of significance in the fermentation of vegetable proteins to produce African food condiments (Omafuvbe *et al.,* 2000).

2.10 Physicochemical changes during kinema fermentation

It has been reported that there was a significant increase in the pH, protein, and reducing sugars and decrease in crude fiber and total sugar content. However, there was no significant change in the level of fat and mineral content. It was also reported a 33 times increase in free fatty acid

value as compared to raw soybean suggesting release of lipase during fermentation process (Poudel, 2018).

2.10.1 Changes in moisture content during kinema fermentation

As fermentation time increased, black soybeans inoculated with *B. natto* exhibited higher moisture and protein content compared with soaked black soybeans (Sarkar and Nout, 2014). The increase in moisture content with fermentation may be due to the moist solid nature of the fermentation and the hydrolytic decomposition of the fermenting substrate by the implicated micro-organisms. However, during traditional *kinema* fermentation the moisture content remain same throughout the period of fermentation (Poudel, 2018).

2.10.2 Changes in pH during kinema fermentation

In alkaline fermentation, protein of the raw material is hydrolyzed into amino acids and peptides, and ammonia is released, which increases the pH of the final products to 8 to 9. The high pH inhibits invasion by pathogenic and spoilage microorganisms (Wang and Fung, 1996). The pH value which was initially 6.94 during 0 h started to decline until 16 h of fermentation time. After that there was rise in pH value 8.51 during 40 h of traditional fermentation of soybeans to produce *kinema.* Hence, during the first 16 h fermentation period, the decrease in mean pH was significant. Then, the pH increased sharply until 40 h (Sarkar and Tamang, 1995). The rise in pH was presumably a result of proteolysis and the release of ammonia following the utilization of amino acids by the fermenting micro-organisms. The release of ammonia is responsible for the ammoniacal odour (Omafuvbe *et al.*, 2000).

Though the titratable acidity (0.1%) in kinema is about 10 times higher than in raw soybeans, the product has a high pH value (>7.8; even up to 8.5). this is due to the high buffering capacity of the legume beans and the proteolytic activities of *B. subtilis* leading to ammonia release which is the characteristic of most vegetable protein fermentation. The increase in proteolytic activity is coincident as the pH drops from an initial 6.9 to 6.4 after 8 h and then rise to 8.6 at the end of fermentation (Sarkar and Nout, 2014).

2.10.3 Changes in Reducing sugar during kinema fermentation

Sugar plays a pivotal role in plants as both nutrient and central signaling or regulatory molecules that modulate gene expression related to plant growth, development, metabolism, stress response, and disease resistance. Reducing and nonreducing sugar play an important role

in the central metabolic pathways and help in the production of secondary metabolites that enhance the medicinal properties of plants (Khatri and Chhetri, 2020). Sucrose is the predominant biochemical component that contributes to the sweetness quality, and the types and amounts of sugars have a direct influence on the organoleptic quality of vegetable soybean. Sucrose, fructose, glucose, glucarate, galactarate, galactose, maltose, and mannose were present in the seeds of vegetable soybean at harvest. Of these free sugars, sucrose presented a significantly higher concentration compared with the other free sugars, followed by fructose and glucose (Song *et al.*, 2013).

One of study from the traditionally fermentation food from soybean known as soy-daddawa showed that moisture content, pH and total free amino acids increased, titratable acidity and reducing sugars increased in the first 24h and decreased gradually, while the total soluble sugar level dropped gradually with fermentation. The total sugar level in the fermenting seeds decreased gradually while α-amylase activity in the fermenting seeds increased rapidly, attained a peak at 48h and then dropped. The utilization of the soluble sugars by the increasing population of fermenting organisms was probably responsible for the steady drop in the total sugar level. As the population of the fermenting organisms became fairly steady in the later stages of fermentation, the soluble sugar level remained fairly steady. α-amylase may have been responsible for the increased level of reducing sugar in the fermenting seed in the early stage of fermentation. The subsequent drop in reducing sugar levels was presumably a result of their being utilized as carbon and energy sources (Omafuvbe *et al.*, 2000).

2.10.4 Changes in Total polyphenol activity during kinema fermentation

Phenolic compounds (also called phenolics) are derived from several biosynthetic precursors including pyruvate, acetate, some amino acids (phenylalanine and tyrosine), malonyl CoA, acetyl CoA through the action of pentose phosphate, shikimate, and phenylpropanoid metabolism pathways. Flavonoids are equally well-known class of frequently occurring phenolics in wholegrains. Major phenolics found in wholegrains are phenolic acids, flavonoids, and tannins. These plant- derived constituents are bioactive and involved in potentiating the redox defense of the body, prevention, and counteracting oxidative stress and reducing free radical-related cellular damage (Adebo and Gabriela Medina-Meza, 2020). The term phenolic acids refers to phenolic compounds having one carboxylic acid group and are mainly divided into two subgroups:

I. Hydrobenzoic acids: gallic acid, p-hydrobenzoic, protocatechuic, syringic and vanillic acids.
II. Hydroxycinnamic acids: caffeic, ferulic, p-coumaric, and sinapic acids

It is well-known that phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma, and flavor and also in providing health-beneficial effects. They also serve in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores (Sengul *et al.*, 2009). A study showed higher bioactive compounds (catechin, gallic acid, and quercetin) after fermentation in a study on ting from fermented sorghum with a concurrent decrease in total flavonoid content (TFC), total tannin content (TNC), and total phenolic content (TPC). Reported decreases in levels of TPC, TFC and TNC were attributed to degradation and hydrolysis of the phenolic compounds, while a corresponding increase in catechin, gallic acid and quercetin was attributed to a release of these bioactive compounds after fermentation with *Lactobacillus* strains (Adebo and Gabriela Medina-Meza, 2020).

The TPC and anthocyanin contents of black soybeans fermented with *B. natto* also increased with fermentation time**.** The increase in phenolic compounds and anthocyanins was attributed to the catalytic action of β- glucosidase produced by the starter organism (Sarkar and Nout, 2014). Polyphenolic substances that serve as reducing agents, metal chelators, and singlet oxygen quenchers make up the majority of antioxidant nutrients. Many traditional fermented foods, like *Natto,* have been shown to have high levels of these chemicals. (Moktan *et al.*, 2008b) assessed the antioxidant activities of *kinema* and cooked non-fermented (CNF) soybeans using four in vitro methods: stable free radical 2,2diphenyl-1-picrylhydrazyl(DPPH) scavenging activity, Fe+++ -reducing power, Fe++ chelating power, and activity in linoleic acid emulsion system. *Kinema*'s total phenol content was 144% higher than raw soybean, and it is a superior free radical scavenger, reducing power, and metal chelating power than CNF soybeans. Soaking and steaming have both been shown to lower the polyphenol and anthocyanin levels of soybeans. However, during the *natto* fermentation of black soybeans, *B. subtilis* fermentation increases total polyphenolic compound and anthocyanins by up to 10% and 250%, respectively.

Kinema had significantly higher contents of total phenolics and flavonoids as compared to unfermented soybeans while an increase in fermentation time decreased. *Kinema* is reported to contain a higher amount of phenolics compared to non-fermented soybean, as the microbial

activities could release bound phenolics to the free forms from the soybean. However, the total phenolic contents in extract could also vary depending on the extraction conditions such as time, temperature and solvent concentration (Katuwal *et al.*, 2023).

2.10.5 Changes in Nutritional composition during kinema fermentation

Alkaline fermentation increases digestibility due to extensive hydrolysis of protein to amino acids and peptides. In many of the alkaline-fermented foods, the vitamin levels, especially riboflavin, are increased. For example, the riboflavin and vitamin B-12 contents of natto are three to five times greater than that in cooked soybeans (Wang and Fung, 1996). Sarkar demonstrated that soaking and heating reduce the thiamine (Vitamin B1) and riboflavin (Vitamin B2) levels of *kinema*. *B. subtilis* fermentation of cooked soybeans, on the other hand, results in higher concentrations of thiamine (5.8 to 8.4 mg/kg), riboflavin (6.8 to 11.6 mg/kg), and niacin (36.4 to 4.8 mg/kg). They proceeded on to state that the presence of *Enterococcus faecium* in *kinema* reduces the levels of all these vitamins considerably.

Several experiments have demonstrated that fermentation of legumes enhances their nutritive value, reduces some antinutritional natural compounds such as phytic acid, trypsin inhibitor, and oligosaccharides, and exerts beneficial effects on protein digestibility and biological value of legumes (Martin-Cabrejas *et al.*, 2004). Soybeans contain an abundant amount of phenolics such as hydroxycinnamic acid, flavonol, diadzin, daidzein, rutin, flavonol, isoflavone, genistein, which are converted to more bioactive and bioavailable form by fermentation. Moreover, fermented soybean products contain peptides having antioxidant, antitumor, antidiabetic, antimicrobial and Angiotensin I converting enzyme (ACE) inhibitory properties (Katuwal *et al.*, 2023).

2.11 Microbiological changes in kinema fermentation

Bacillus subtilis, Enterococcus faecium are the major microflora reported to be associated in kinema fermentation as bacteria whereas *Candida paraplosis* and *Geotrichum candidum* as yeast. *Bacillus subtilis* being the most dominant organism during the *kinema* fermentation, the completion of fermentation is generally indicated by appearance of white viscous mass on the soybean seeds and typical kinema flavors with a slight odour of ammonia. The duration of fermentation is reported to be 2-3 days depending on weather condition (Tamang, 2009).

A number of interesting changes occurs during soybean fermentation.The high initial load of *B. subtilis*, even at the onset of fermentation, was due to presence on raw soybeans (Sarkar *et al.*, 1994). The high initial count of *B. subtilis* increased significantly at every 8 h interval during 48 h fermentation period. Its load increased from 10 cfu g^{-1} (wet weight) of soybeans at 0h to 109 cfu g^{-1} (wet weight) of kinema produced at 18 h. The increase in mean cfu of *E. faecium* was significant at every 8 h interval until the first 40 h of fermentation. Although the load of the only yeast, *C. parapsilosis* recovered from the laboratory made samples, was much less compared with the bacterial load, the mean cfu of the yeast cells increased throughout the period of fermentation; until 32 h, the increase was significant at every 8 h interval (Sarkar and Tamang, 1995b). Spores of *B. subtilis,* which are normal inhabitants of soybeans, pass through soaking and cooking treatments to initiate and carry out fermentation. The mean viable counts of *B. subtilis, E. faecium* and C. *parapsilosis* increase in fermenting beans. In monoculture fermentation, the count of B. *subtilis* cells increase from an initial 10^5g up to 10^9g wet beans after 48 h at 37°C.

2.12 Release in Bioactive constituents during kinema fermentation

2.12.1 Changes in Free radical scavenging activity during kinema fermentation

Antioxidants can be defined as substances that, when present in food, delay, control, or inhibit oxidation and deterioration of food quality. Similarly, it also reduces the risk of degenerative diseases arising from oxidative stress (Halliwell, 1999). Antioxidants have been reported to prevent oxidative damage caused by free radical which can interfere with the oxidation process by reacting with free radicals, chelating, and catalytic metals and also by acting as oxygen scavengers (Shahidi *et al.*, 1992). It has been reported that soybeans contain numerous bioactive compounds which inhibit low-density lipoprotein oxidation, scavenge free radicals, and reduce the incidence of DNA damage by cyclophosphamide. The study showed higher in vitro antioxidant activities of the fermented black soybeans were found in DPPH than soaked and steamed soybeans. This may be due to increased total polyphenol content and isoflavone compositional changes during fermentation (Hu *et al.*, 2010).

A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) which is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity (Leaves and Leaves, 2014). The DPPH assay method is based on the

reduction of DPPH, a stable free radical. The free DPPH radical with an odd electron gives absorbance (purple color) at 517nm. When the antioxidants in plant extract react with DPPH, it reduces to DPPH-H and results in decolorization to yellow color with respect to the number of electrons captured. The color absorbance corresponds inversely to the radical scavenging activity of the sample extract. The scavenging of DPPH by radical scavengers can be summarized as:

DPPH * + FE ⟶ DPPH-H +A …… (1)

DPPH +A* ⟶ DPPH – A ……. (2)

A* +A * ⟶ A – A ……... (3)

collision of radicals with abstraction of an atom by one radical from another equations (Leaves and Leaves, 2014).

Methanol extract of the soaked black soybean, cooked black soybean, and fermented black soybean, cooked black soybean, and fermented black soybean for 48 h showed a DPPH scavenging effect of the methanol extract of all samples increased as the concentration increased. Methanol extract of the soaked black soybean, cooked black soybean, and fermented black soybean for 48 h showed a DPPH scavenging effect of 30.38%, 28.78% and 78.22% respectively, when dosage was raised to 5 mg/ml. among all the extracts tested, the samples fermented by *B. natto* for 48h at a concentration of 15 and 20 mg/ml respectively, exhibited the strongest final scavenging effect, with 92.24% and 94.34%. DPPH free radical in the clear, the fermented black soybean natto products had remarkably stronger than the unfermented, steamed soybeans (Sarkar and Nout, 2014).

In the present study, higher in vitro antioxidant activities of the fermented black soybeans were found in DPPH than soaked and steamed soybeans. This might be mainly due to increased total polyphenol content and isoflavone compositional changes during fermentation. Total polyphenol content increased to 1.5-fold during the 3-day fermentation. It was found that isoflavones have direct free radical quenching ability, with genistein and daidzein being particularly effective (Hu *et al.*, 2010). It was also reported that, during fermentation, at least a partial cleavage or change in the glycosides takes place, with increase in glycosidase and glucuronidase activities, releasing potent antioxidant substances by transformation of

flavonoids (McCue and Shetty, 2003). IC50 concentration for DPPH radical scavenging activity decreased initially approaching minimum value at 35.5 h followed by gradual increase to attain maximum value at 58.5 h and finally decreasing at a longer fermentation time. This may be due to the fact that during fermentation some phenolic compounds undergo hydrolysis and degradation while the others such as catechin, gallic acids, quercetin, ferulic acid etc. could accumulate. Since the phenolic compounds are antioxidants that scavenge free radicals, this could account for the initial increase in antiradical activity with fermentation time (Katuwal *et al.*, 2023).

2.13 Dietary significance of kinema and culinary

Fermented foods and beverages have historically been an integral part of the human diet and have long been thought to provide health benefits. Potential health benefits of fermented foods include a reduced risk of hypertension, diabetes, obesity, high cholesterol, diarrhea, thrombosis, and so on. Melatonin is synthesized, as well as GABA, which regulates blood pressure and protects against cardiovascular disease and cancer. Exopolysaccharides, which have cholesterol-lowering, immunomodulator, antioxidant and anti-cancer properties, are generated, and a variety of bioactive peptides such as anti-hypertensive, anti-cancer, anti-microbial, anti-adipogenic, anti-mutagenic, anti-thrombotic, and anti-atherogenic peptides are produced. Fermentation is an old food processing technique that has been adopted for centuries around the worlds, especially in developing nations. This process is generally completed through microbial actions, which positively alter the appearance, flavor, functionalities, nutritional composition, color, and texture. The fermentation process itself yields beneficial effects through direct microbial action and production of metabolites and other complex compounds (Adebo and Gabriela Medina-Meza, 2020).

Fermentation has also been proven to improve the nutritional value of soybean by increasing the bioavailability of nutrients and reducing anti-nutritional factors. Moreover, fermented soybean can be used as a functional ingredient with high protein digestibility and as a good source of probiotics. Several studies have proved that fermentation with microorganisms may reduce the immunoreactivity of soybean proteins (Yang *et al.*, 2020). Reports have demonstrated the antidiabetic effects of fermented soybean products in type 2 diabetes. Increasing evidence has linked this health promoting effect to its bioactive peptides such as isoflavonoids and bioactive peptides. Also, a study has shown that fermentation of legume beans increases both the polyphenol content, which is due to the release of free polyphenol

aglycones that are not readily available from their glycosides and peptide fractions; due to hydrolysis of native protein by the fermenting micro- flora (Ademiluyi *et al.*, 2014).

2.13.1 Antihypertensive activity/ACE inhibition activity

Angiotensin I converting enzyme (ACE), is the key enzyme in the renin-angiotensin system, is associated with the regulation of blood pressure. ACE increases blood pressure by both converting the inactive angiotensin I to the potent vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin. It has long been thought that eating natto is useful for preventing an increase in blood pressure. An ACE inhibitor partially purified from natto was orally administered as a single dose in spontaneously hypertensive rats, and the blood pressure was measured. The administration of the inhibitor, even at the lowest dose, resulted in a significant decrease in blood pressure 4 h after administration. Hence. The ACE inhibitor from natto appears to moderately reduce blood pressure relative to increase in dosage (Ibe *et al.*, 2009). Miso, a fermented soybean paste, is a mixture of soybeans that contains significant amounts of vitamins, minerals, fat, salt, carbohydrates, vegetable protein and microorganisms. In humans, miso acts as a scavenger reactive oxygen species, an estrogen-like substance and an ACE-inhibitor (Şanlier *et al.*, 2019).

2.13.2 Anti diabetic activity

Diet is associated with type 2 diabetes through changes in the gut microbiota. Fermented soybean foods contain a variety of health-beneficial components, and their continuous intake improves dysbiosis, which in turn promotes the production of gut microbiota-related metabolites, including short chain fatty acids and bile acids. Many fermented soybean products improves blood glucose and lipid levels by altering the gut microbiota, leading to the inhibition of cholesterol synthesis, promotion of lipolysis, increased antioxidative capacity, and lowered reactive oxygen species levels (Hashimoto *et al.*, 2023). Nattokinase (NK), which is an enzyme produced by *Bacillus subtilis* in the fermented soybean, possesses a strong anti-hyperlipidemic activity, which can prevent atherosclerosis. It was also reported that natto was effective in reducing cholesterol and its associated lipids. NK prevents atherosclerosis through its antioxidant property, which results in the reduction of lipid peroxidation and improved lipid metabolism (inhibition of low-density lipoprotein LDL oxidation (Gopikrishna *et al.*, 2021).

2.13.3 Antimicrobial activity

Antimicrobial activity of the *Bacillus* species isolated from the various traditional FSF like *Cheonggukjang, doenjang*, and *meju* that produces antimicrobial compounds like proteins, enzymes, lipoproteins, and bacteriocins. The lipoproteins present in fermented soybean products have found to show anti-adhesive effect against several bacterial pathogens such as *Botrytis cinereal, Fusarium moniliforme, Micrococcus luteus and S. typhimurium* and food-borne pathogens like *Bacillus cereus*. Hence, these antimicrobial peptides could be used as an alternative to antibiotics for (Gopikrishna *et al.*, 2021) the treatment of bacterial/ fungal diseases as well as food preservatives.

2.13.4 Anticancer effect

Environmental factors, especially diet, are considered to play a key role in carcinogenesis. The incidence of cancer in the Asian population is relatively low, and Asians traditionally consume large amounts of soy-based foods, which are rich in isoflavones. Soybean isoflavones are believed to have the potential to reduce cancer risk through their antioxidant activity and estrogen-like structure. Mostly, the anticancer effects of fermented soy products are associated with isoflavones. Genistein is said to have a broad- spectrum anticancer effect on cancers of the breast, prostate, esophagus, pancreas, stomach, and colon and metacarcinoma, lymphoma and neuroblastoma (do Prado *et al.*, 2022).

Part III

Materials and methods

3.1 Materials

3.1.1 Collection of Soybean

The local variety of brown soybean was collected from the local market of Dharan (26.8065° 74N, 87.2846° E). The collected grains were cleaned and sorted to remove foreign materials and damaged ones.

3.1.2 Chemicals

Phosphate buffer, casein solution, Tricholoro Acetic acid (TCA), Sodium Carbonate ($Na_2CO_{3)}$ reagent, Phenol reagent, Barford reagent, Dinitrosalycylic acid (DNS) reagent, Starch, Barium hydroxide Ba $(OH)_2$, Zinc sulfate ($ZnSO_4$), Sodium hydroxide (NaOH), Folin-Ciocalteu (FC)reagent, Methanol, DPPH

3.1.3 Glassware

Test tubes, micropipette, volumetric flask, conical flask, measuring cylinder, micropipette, funnel, watch glass and petri dish

3.1.4 Equipment

Electric balance, Hot air oven, Incubator, Hot water bath, Spectrophotometer, centrifugation machine, Weighing machine, magnetic stirrer, Refrigerator, Bunsen burner, Autoclave, Rotatory Shaker, heating mantle

3.1.5 Other requirements

Filter paper, mortar and pestle, bamboo baskets, muslin cloth, banana leaves, tray etc.

3.2 Methods

3.2.1 Preparation of kinema

This method describes a traditional preparation of *Kinema*, a fermented soybean product, with slight modifications from the original method given by Tamang (2015). Here's a step-by-step summary:

- **Sorting and Cleaning**: Brown soybeans are sorted manually to remove any non-soybean materials such as stones, straw, or dirt, and are thoroughly cleaned.
- **Soaking**: The cleaned soybeans are soaked in water overnight to soften them.
- **Flaking and Autoclaving**: After soaking, light flaking is performed on the soybeans. They are then autoclaved at 15 psi for 30 minutes to sterilize and soften them further.
- **Cooling and Ash Addition**: The autoclaved soybeans are cooled to approximately 40°C. Afterward, 1% firewood ash is added. The firewood ash helps create the right pH conditions for fermentation.
- **Incubation**: The cracked soybeans are transferred to a bamboo basket lined with banana leaves. The basket is covered with a muslin cloth, and the beans are incubated at 30°C for 72 hours to allow fermentation.

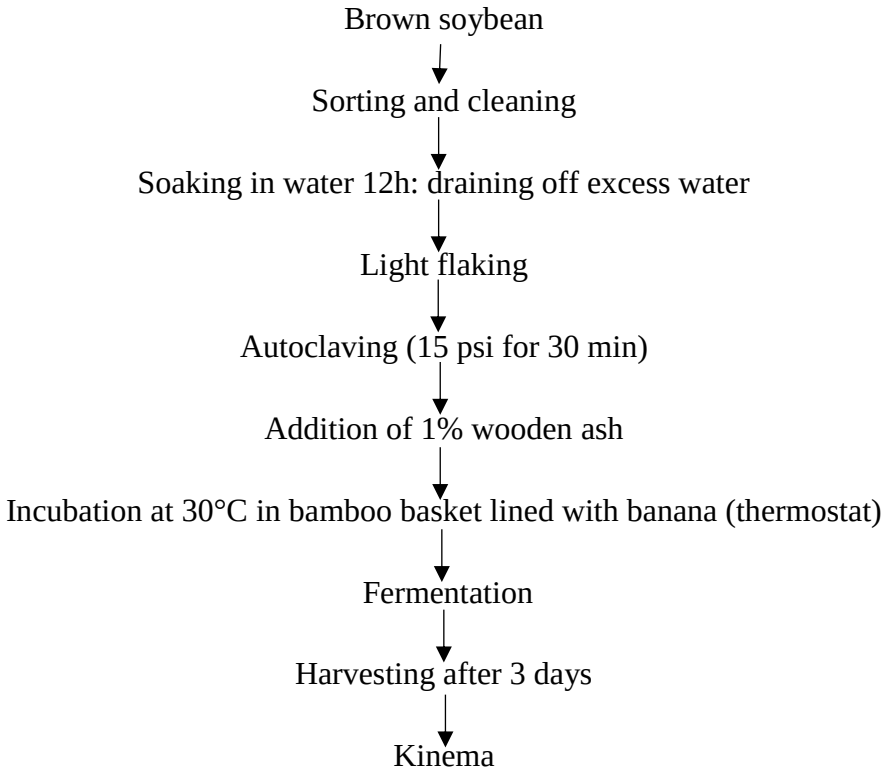


Fig. 3.1 preparation of kinema

Source: Tamang (2015)

3.2.2 Research frame work

Soybeans were winnowed, cleaned, and then ground into powder for proximate analysis (e.g., moisture, protein, fat, ash, and carbohydrate content) before fermentation. Samples were prepared at different fermentation intervals to monitor changes over time.

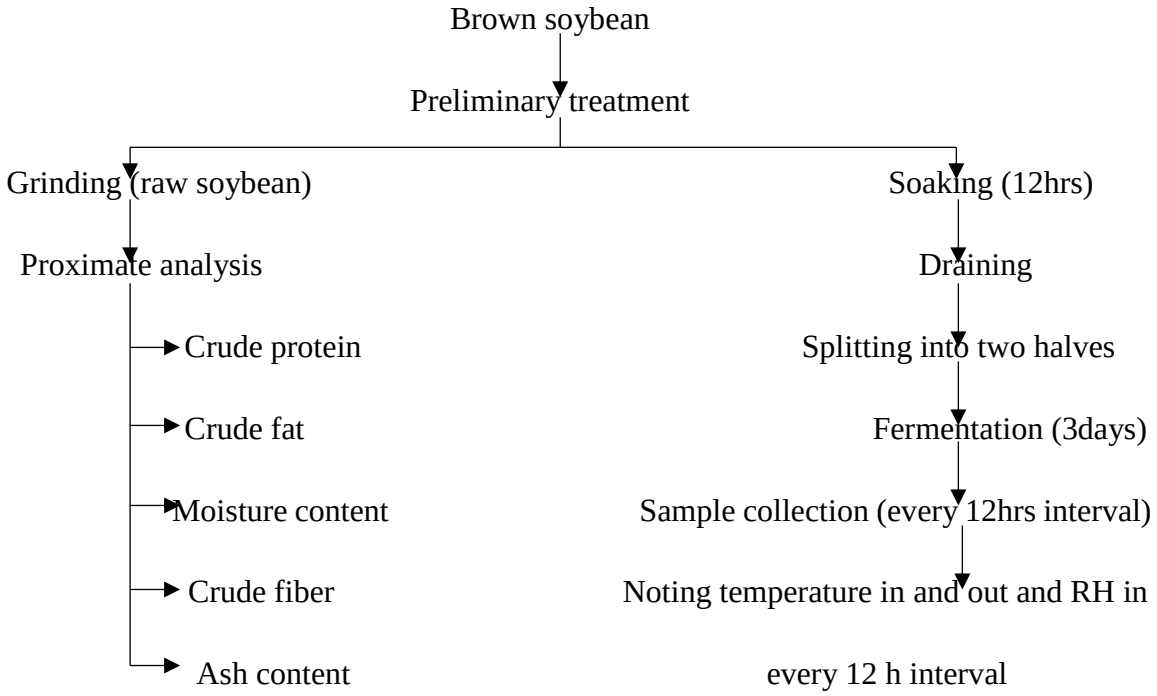


Fig 3.2 Flow diagram of sample collection and proximate analysis

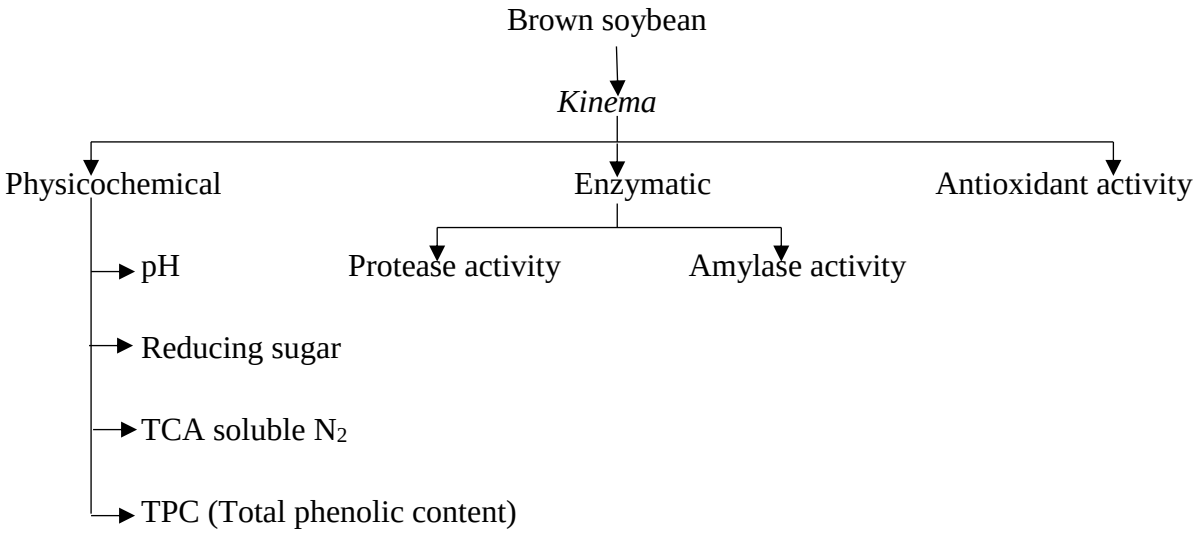


Fig: 3.3 Flow diagram for the analytical procedure

A total of **7** samples were taken, each corresponding to a specific fermentation duration (0, 12, 24, 36, 48, 60, and 72 hours). After each fermentation time point, the *Kinema* was collected and stored in refrigeration for further analysis. The aim of research was to examine changes in various characteristics (e.g., reducing sugars, TPC, enzymatic activity, antioxidant etc.) over the fermentation period, as shown in fig. 3.2 and 3.3.

3.3 Physicochemical analysis

3.3.1 Moisture content

The gravimetric method was employed to measure moisture content (Ranganna, 1986). The sample was heated in an oven at **103 ± 2°C** until it reached a constant weight. The sample was placed in tared petri plates and the weighing was performed. The moisture content is expressed as a percentage of the original sample weight.

3.3.2 Crude protein content

Crude protein content of the samples was determined indirectly by measuring total nitrogen content by micro Kjeldahl method. Factor 6.25 was used to convert the nitrogen content to crude protein. The calculated data were presented per 100 g on dry basis (Ranganna, 1986).

$$\%\ \text{Nitrogen} = \frac{(\text{sample titre-blank titre}) \times \text{Normality of HCl} \times 14 \times 100}{\text{wt. of sample} \times 100}$$

$$\%\ \text{protein} = \%\ \text{nitrogen} \times 6.25$$

3.3.3 Crude fat content

Crude fat content of the samples was determined by solvent extraction method using Soxhlet apparatus and solvent petroleum ether. The calculated data were presented as gram per 100 g on dry basis (Ranganna, 1986).

$$\%\ \text{Crude fiber} = \frac{(\text{wt. of ether soluble materials}) \times 100}{\text{wt. of sample}}$$

3.3.4 Total ash content

Total ash content of the samples was determined by following the method given by Ranganna using muffle furnace (Ranganna, 1986).

$$\% \text{ Total ash} = \frac{\text{wt. of ash } \times 100}{\text{wt. of sample taken}}$$

3.3.5 Crude fiber content

Crude fiber content of the samples was determined by the chemical process, the sample was treated with boiling dilute Sulphuric acid, boiling sodium hydroxide and then with alcohol as standard method given in (Ranganna, 1986). The calculated data were presented as g/100 g on dry basis.

$$\% \text{ Crude fiber} = \frac{\text{loss in wt. noted } \times 100}{\text{wt. of sample taken}}$$

3.3.6 Carbohydrate

The carbohydrate content of the sample was determined by difference method which is given below;

% Carbohydrate (dry basis) =100- (% protein-% fat-% ash-% crude fiber)

3.4 Preparation of *kinema* crude enzyme extract

10 gm of *Kinema* was taken from each fermentation interval (0, 12, 24, 36, 48, 60, and 72 hours) and ground using a mortar and pestle. 20 ml of sodium phosphate buffer (1:2 ratio, pH 7, 50 mM) was added to the ground sample to facilitate enzyme extraction. Stirring was done to ensure proper mixing and extraction of enzyme. Stirring was performed under refrigeration temperature to prevent the denaturation of enzyme. The mixture was filtered using muslin cloth that contained the crude enzyme extract. The filtrate was then transferred into centrifuge tubes for centrifugation (5000 rpm for 6-7 minutes) to separate the enzyme-containing supernatant from any remaining solid particles. A final filtration was performed using filter paper (Whatman no. 41) to ensure the purity of the crude enzyme extract, which was collected and kept in refrigeration for further analysis (Khadka *et al.*, 2024).

3.4.1 Determination of Protease activity during Kinema fermentation

Protease assays were carried out using casein as the substrate in accordance with the instructions provided by (Khadka *et al.*, 2024) with slight modifications. 0.2 ml of crude extract was added to 2.2 ml of (2 mg/ml) casein solution prepared with sodium phosphate buffer (50Mm, PH 7). The reaction was incubated at temperature 35°C for 20 min and the reaction was further stopped by the addition of 3.5 ml of 5% Trichloroacetic acid (TCA). Similarly, it was then cooled by refrigerating and later centrifuged at about 7000rpm for 7 min. Filtration of the supernatant was done by using Whatman no.41 filter paper and collected in a clean test tube. In the similar way, the blank was also prepared by addition of same amount of enzyme extract after addition of 5% TCA which was then followed by incubation, refrigerated, centrifuged and lastly filtered. When protease digest the casein, tyrosine gets liberated along with other amino acids and peptide fragments. Hence, the tyrosine liberated was determined as protease activity after the reaction with Folin and ciocalteus phenol reagent. In this process, each 1 ml filtrate of blank and test sample were mixed with 5 ml of 2% Sodium carbonate reagent and 0.5ml FC-reagent in separate test tubes.

The mixed reagents and samples were incubated at 37°C for 30min and the absorbance was measured at 700nm by spectrophotometer. A standard curve was made by reacting known amounts of tyrosine with Folin Ciocalteu reagent, and the absorbance values generated by the protease activity were compared to the standard curve. This makes it easier to correlate changes in absorbance to the number of micromoles of tyrosine. The amount of tyrosine equivalents released from casein per minute in micromoles of tyrosine. The amount of tyrosine equivalents released from casein per minute in micromoles, or Units, was used to quantify the activity of the protease samples. At 37°C and 7.0 pH, on protease unit is the volume of casein digested to give color equal to 1.0 micromole (181g) of tyrosine per minute. The formula was used to determine the protease activity (unit/ml enzyme extract)

$$\text{Protease activity (Units/ml enzyme)} = \frac{(\mu\text{mol tyrosine equivalent}) \times V_t}{V_e \times V_c \times t \times 181}$$

Where, 181 = mol. wt of tyrosine

Vt =total assay volume

Ve =Enzyme volume

Vc = vol. of TCA filtrate

t = time of hydrolysis in min

3.4.2 Determination of Amylase activity during kinema fermentation

The α-amylase activity of the enzyme extract was determined using starch as the substrate. The reaction mixture consisted of 1 ml of 1% starch solution (pH 7) and 0.5 ml of enzyme extract, which were placed in tubes. The tubes were incubated at 37°C for 30 minutes. After incubation, 1 ml of DNS reagent was added, and the tubes were heated at 95°C in a water bath for 10 minutes, then covered with foil. The tubes were cooled in a refrigerator, and 2 ml of distilled water was added. Following the addition of 0.5 ml of 0.5% Na_2SO_3, the absorbance was measured at 540 nm (Salim *et al.,* 2019).

3.4.3 Determination of protein content during Kinema fermentation

The Bradford method was used to determine the protein content of the crude extract and the partly purified enzyme fraction (Bradford, 1976). Following Kruger (2009), the Bradford assay was performed by adding 3 ml of Bradford reagent to 100 µl of each protease extract, mixing thoroughly with a vortex mixer, incubating at 37°C for 30 minutes, and measuring the absorbance at 595 nm. A standard curve was generated using bovine serum albumin (BSA) solutions ranging from 0.1 to 1.2 mg/ml. The protein concentration of the samples was calculated by comparing their absorbance to the BSA standard curve and expressed as mg per ml.

3.5 Preparation of Methanol extract

2 grams of the sample were ground with 16 ml of 80% methanol, and 4 ml of distilled water was added in a conical flask. The mixture was placed on a rotary shaker for about 1 hour, followed by filtration. After centrifugation for 15 minutes, the filtrate was transferred into a beaker and filtered again. The extract was then covered with foil and stored in a refrigerator.

3.5.1 Determination of total phenolic content (TPC) during Kinema fermentation

The total phenolic content of the *kinema* was investigated by Folin Ciocalteu procedure as described by (Jaradat *et al.*, 2015). Gallic acid was used as the standard, and the total polyphenols were expressed as mg/g Gallic acid equivalents (GAE) based on the calibration curve ($R^2 = 0.9562$).

To prepare standard solution, 20 mg of Gallic acid was accurately weighed into a 10 ml volumetric flask and dissolved with 10 ml of 80% methanol. Standard curve solutions of 0, 30, 60, 120, 180, 240, and 300 µl were prepared by adding methanol to give a final volume of 300 µl, corresponding to 0, 100, 200, 400, 600, 800, and 1000 µl/ml of Gallic acid.

In the assay, 300 µl of each sample was mixed with 3.6 ml of 2% sodium carbonate and 300 µl of a 1:1 diluted Folin-Ciocalteu reagent in test tubes. The tubes were covered with aluminium foil and allowed to stand for 30 minutes at room temperature. The absorbance was then read at 750 nm using a UV/Vis spectrophotometer. Each sample was prepared in triplicate, and the readings obtained were noted (Jaradat *et al.,* 2015).

3.5.2 Determination of antioxidant activity during kinema fermentation

A method describe by Vignoli *et. al.,* (2011) with slight modifications was used to assess the DPPH radical scavenging activities. 200 µl of the phytochemical extraction solution was collected and mixed with 3 ml of DPPH methanol (0.004%) solution. After incubated at 37°C in the dark for 20 minutes, the absorbance of the mixture was measured at 517nm using a UV-Vis spectrophotometer. The radical scavenging effect (percent) was calculated using the following equation:

$$\text{Radical scavenging activity} = \frac{\text{c-s}}{\text{c-b}} \times 100\,\%$$

Where, c = Absorbance of control, s = Absorbance of sample and b = Absorbance of blank

3.6 Determination of reducing sugar

Four grams of the sample was ground and mixed with 40 ml of distilled water. The mixture was then placed in a rotary flask shaker for 1 hour to ensure thorough mixing. After shaking, the solution was filtered using muslin cloth, and volume was adjusted to 40 ml. The filtrate was then, centrifuged (5000 rpm) for 5 minutes. After centrifugation, the supernatant was filtered using Whatman No. 41 filter paper and the volume was again adjusted to 40 ml. 8 ml of the prepared sample was taken for analysis.

The sample was mixed with a combination of 0.5 ml $Ba(OH)_2$ (barium hydroxide) and 0.5 ml $ZnSO_4$ (zinc sulfate) to precipitate interfering substances (proteins). The mixture was allowed to stand for 5 minutes and filtered to remove any precipitate. To the filtered solution,

2 drops of 0.1 N NaOH were added to neutralize any remaining acidity. Finally, the volume was made up to 10 ml using distilled water, making it ready for the reducing sugar determination process.

The reducing sugar was determined using DNS method with slight modifications. Under alkaline condition, 3,5-dinitrosalicylic acid (DNS) solution is reduced by reducing sugars to 3-amino-5-nitrosalicylic acid. The intensity of the dark-red color formed as a result of the reaction was measured against standard at 510nm. 0.5 ml of sample solution was added in the test tubes and then 1 ml of DNS reagent is added. It was then heated in water bath for 10 mins. The tubes were covered with foils. 0.5 ml of sodium sulphite and 1 ml distilled water was added for the dilution. The tubes were cooled and the absorbance of the dark-red/orange-red color was read at 510 nm. A series of standards using glucose was run and a graph was plotted of absorbance versus reducing sugar content (KC and Rai, 2007).

3.7 Determination of TCA soluble Nitrogen

TCA-N was determined according to the method of Ketnawa and Ogawa (2019), with slight modifications. Sample extraction was done with the similar procedure as for the determination of reducing sugar. After that homogenization of the sample (3ml) with 3 ml of 10% TCA was carried out. After centrifugation for 7 min, the supernatant was filtered through Whatman paper. 5 ml of sodium carbonate was added in 0.5 ml filtrate which was diluted by 2 times. It was kept in room temperature 10 min, 0.5 ml FC reagent was then added. The reading was taken at 700 nm.

3.8 Determination of pH change during kinema fermentation

Soybeans were powdered into fine particles using a mortar and pestle. A 10% solution was made by adding distilled water to the powdered soybeans. The mixture was placed in a rotary shaker for 1 hour to ensure thorough mixing and extraction. After the shaking period, the solution was filtered to remove any solid particles, leaving a clear solution for pH measurement. The pH meter was calibrated using buffers of known pH values to ensure accuracy. This step ensures the pH readings are reliable. A glass electrode from the calibrated digital pH meter was inserted into the filtered soybean solution. The pH of the solution was then measured and recorded.

3.8 Statistical Analysis

Analysis was carried out in triplicate. Data on analysis of proteolysis activity, amylase activity, protein, TPC, DPPH, TCN soluble nitrogen and reducing sugar in different fermentation hour was tabulated for comparison and line chart were drawn using Microsoft Excel 2021. Data was statistically processed by GeneStat version 12.1.0.3338 for analysis of variance (ANOVA). Means of data were compared by post hoc method at 5% level of significance.

PART IV

Result and discussion

The study involved analyzing various biochemical parameters during the fermentation process, which was observed at 12-hour intervals over a 72-hour period, analyzing the protein content, enzyme (protease and amylase) activity, antioxidant activity, total phenolic content, reducing sugar, TCA soluble nitrogen and pH change during fermentation. The results obtained from the study are presented and discussed on the following headings.

4.1 Proximate composition of soybean

The result obtained from the proximate analysis of raw brown soybean and kinema fermented from these soybeans are presented in table 4.1.

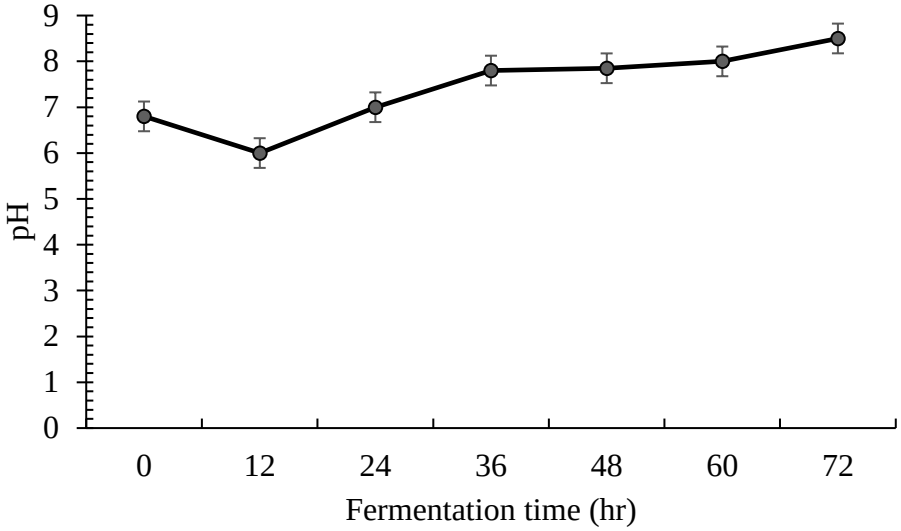
Parameters	**Brown soybean**	**Brown Kinema (after 3 days fermentation)**
Moisture (%)	10.5 ±0.2	63.40±0.114
Crude protein (%) (db)	42.87±0.232	46.50±0.125
Crude fat (%) (db)	18.46±0.204	23.20±0.421
Total ash (%) (db)	5.32±0.124	5.90±0.145
Crude fiber (%) (db)	3.2±0.112	3.40±0.121
Carbohydrates (%) (db)	30.15±0.387	21±0.812

*Values are expressed in mean of triplicate analysis ± S.D.

4.2 Effects of fermentation time on pH

The pH is an important indicators of the fermentation process, as its changes are the consequent of metabolic activity of microorganism (Adebo *et. al.*, 2018). Samples were prepared with a 12-hour interval during *kinema* fermentation over a span of 3 days to determine changes in pH over time. Initially, the pH was measured at 6.8, low pH could be attributed to the production of organic acids from free sugars (Chun *et al.*, 2020). Over the course of 12 and 24 hours, the

pH gradually increased to 6.0 and 7.0 respectively. Subsequently, the pH continued to rise gradually, reaching 7.8 at 36 hours, 7.85 at 48 hours, and 8.0 at 60 hours, similar result was reported by (Cho *et. al*., 2011). By the third day, the pH had further increased to 8.5, as illustrated in Fig. 4.1. The increase in pH observed towards the end of fermentation is likely due to the formation of biogenic amines (Chun *et al*., 2020), and proteolysis and release of ammonia following the utilization of amino acid by fermenting micro-organism (Omafuvbe *et al*., 2000) . Hydrolysis of protein to produce amines and ammonia through peptides and amino acids is by *B. subtilis* responsible for the final change in pH (Sarkar and Tamang, 1995a)



*Values are expressed in mean of triplicate analysis ± S.D.

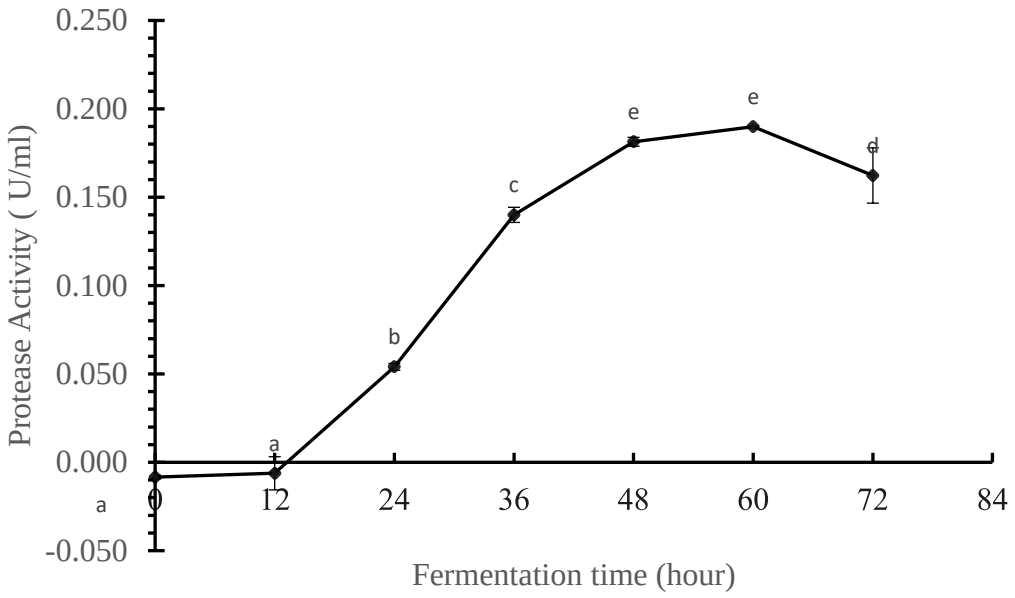
Fig 4.1 Effect of fermentation time on pH

Unlike other fermented soybean products, *kinema* involves adding 1% wood ash with an alkaline pH to the fermenting soybean grits. This addition is to increase the product's alkalinity (pH > 7.0) and to introduce beneficial microbiota present in the wood ash (Kharnaior and Tamang, 2021). Similarly, the changes in pH values is a very important indicator representing microbial activity during fermentation.

4.3 Effects of fermentation time on Protease activity

Seven samples with 12 h fermentation time difference was taken under study for the protease activity. The study showed that protease activity was negative initially (0 and 12hrs) and increased after 12hrs of fermentation and peaked the activity at 60 h of fermentation. Study

reported by Allagheny *et. al*., (1996); Sarkar *et. al*., (1913) showed that maximum proteolytic activity reach after 12-24 hr of fermentation due to higher activity of *B. subtilis*. The protease activity slowly declined when the fermentation time increased to 72 h as shown in fig. 4.2.



*Values are expressed in mean of triplicate analysis ± S.D.

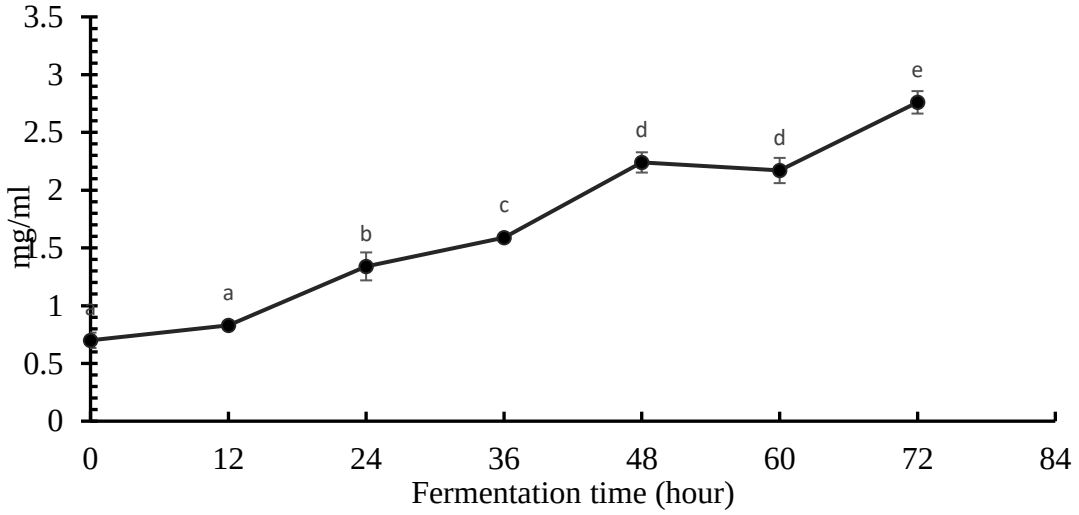
Fig 4.2 Effect of fermentation time on protease activity

Among the 7 samples, there was a negative activity at 0 and 12 h with the value of -0.008 and -0.006 U/ml respectively. A sudden increase occurred after 12h peaking the value to 0.190± 0.001 U/ml at 60 h of fermentation and declined as the fermentation time increased as shown in fig 4.2, similar pattern of increase and decrease of enzyme activity with fermentation time was reported in *doenjang* (Korean fermented soybean paste) by (Choi *et. al*., 2007). Rapid increase in total free amino acids in early phase of fermentation causes increase in protease activity. The protease activity drops with increase in fermentation time while free amino acid level increases steadily (Omafuvbe *et al.*, 2000). Protease activity was not significantly ($p>0.05$) different between the fermentation time of 0 and 12h, Protease activity at fermentation time 24 (0.055 U/ml) 36 (0.138 U/ml) and 72 (0.162 U/ml) h was significantly different ($p<0.05$) with fermentation time of 0, 12, 48 and 60 hrs. Protease activity was not significant different between the fermentation time 48 h (0.181 U/ml), 60 h (0.189 U/ml). High protease activity is the result of increase in water-soluble nitrogen produced by *B. subtilis* with increase in fermentation time (Tamang and Nikkuni, 1996).

In many fermented product *B. subtilis* has shown to increase with fermentation. So, it can be expected that *B. subtilis* plays the important role in secretion of protease enzyme. The result indicated that *kinema* isolated organism (*Bacillus subtilis)* probably played an essential role in protein degradation. Proteases secreted by *B. subtilis* growing on soybean surface might hydrolyze soy proteins to oligopeptides and amino acids. Hence, the proteolytic activity increase and the protein concentration gets decreased during the *kinema* fermentation (Visessanguan *et al.*, 2005).

4.3 Effects of fermentation time on protein

The change in protein content of fermented soybean during kinema fermentation were analyzed in different time interval (0, 12, 24, 36, 48, 60 and 72) hour. The overall increase in protein content with respect to fermentation time is demonstrated in the fig 4.3.



*Values are expressed in mean of triplicate analysis ± S.D.

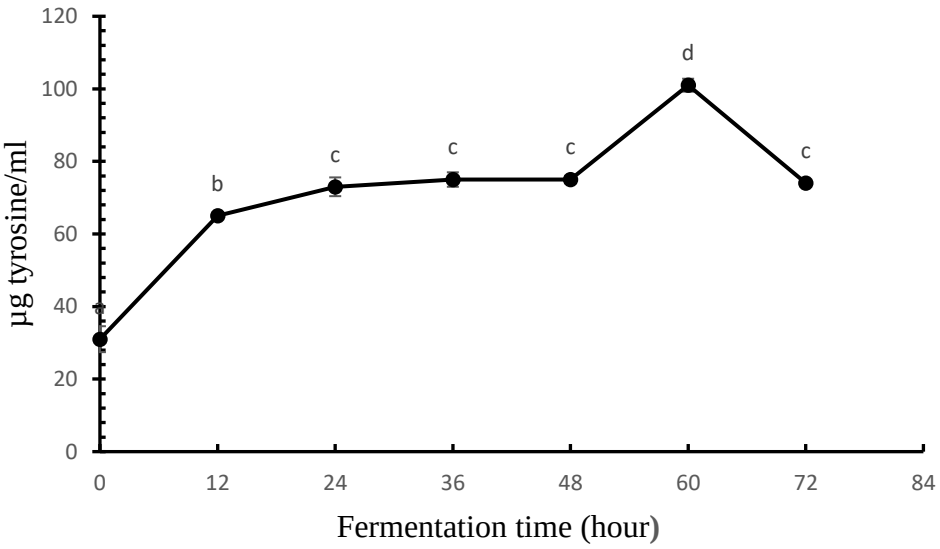
Fig 4.3 Effect of fermentation time on protein content

The protein content gradually increased resulting highest protein content 2.758 ± 0.097 mg/ml at 72 h of soybean fermentation. The mean value of protein content in raw soybean was found to be 0.702 ±0.066 mg/ml. Protein content was increased on the progressive interval of fermentation hour. The mean value was 0.831 ± 0.030 mg/ml, 1.339 ± 0.121 mg/ml, 1.589 ± 0.022 mg/ml and 2.242 ±0.088 mg/ml on different fermentation time (12, 24, 36 and 48) h respectively. On the contrary, slight decrease was observed and accounted to 2.171 ±0.109

mg/ml at 60 h. However, the value suddenly increased and peaked at 72 h of soybean fermentation. The analysis of variance (Appendix E) showed that there was significant difference ($P<0.05$) between 72 h with other fermentation hour (0, 12, 24, 36, 60 and 48). However, there was no significant difference ($P>0.05$) between raw soybean and 12 h sample and also between 60 h sample and 48 h sample.

After 48 hours of fermentation, protein content in all the varieties of *kinema* was found to increase. The increase in protein content could be due to the increased amount of nitrogen, as nitrogen fixing capacity of *Bacillus subtilis* has been observed. It was reported that the ability of *Bacillus subtilis* to secrete high level of protein into the growth media and also observed the significant increase in crude protein during fermentation by *Bacillus subtilis*. During *kinema* fermentation, there was nearly an increase in protein by 3-6% as compared to unfermented soybean (Nepali, 2007).

4.4 Effects of fermentation time on TCA soluble Nitrogen



*Values are expressed in mean of triplicate analysis ± S.D.

Fig. 4.4 Effects of fermentation time on TCA soluble nitrogen

It was observed that the TCA soluble nitrogen was increasing with every 12 h difference starting from 0 h and reached its peak at 60 h 101.382 ± 1.786 µg tyrosine equivalent/ml however, the value dropped and reached 74.345 ± 2.585 µg tyrosine equivalent/ml at 72 h. There was a upward trend where the mean TCA soluble nitrogen was given as (31.291 ± 3.575,

64.952 ± 0.516, 73.345 ± 2.585, 75.333±1.993) µg tyrosine equivalent/ml at (0, 12, 24 and 36) h as shown in fig 4.4. The value then slightly decreased and reached 74.6 ± 1.056 µg tyrosine equivalent/ml at 48 h of soybean fermentation. Statistical analysis at 5% level of significance shows that the TCA soluble nitrogen observed at 60 h among was significantly different from different time intervals.

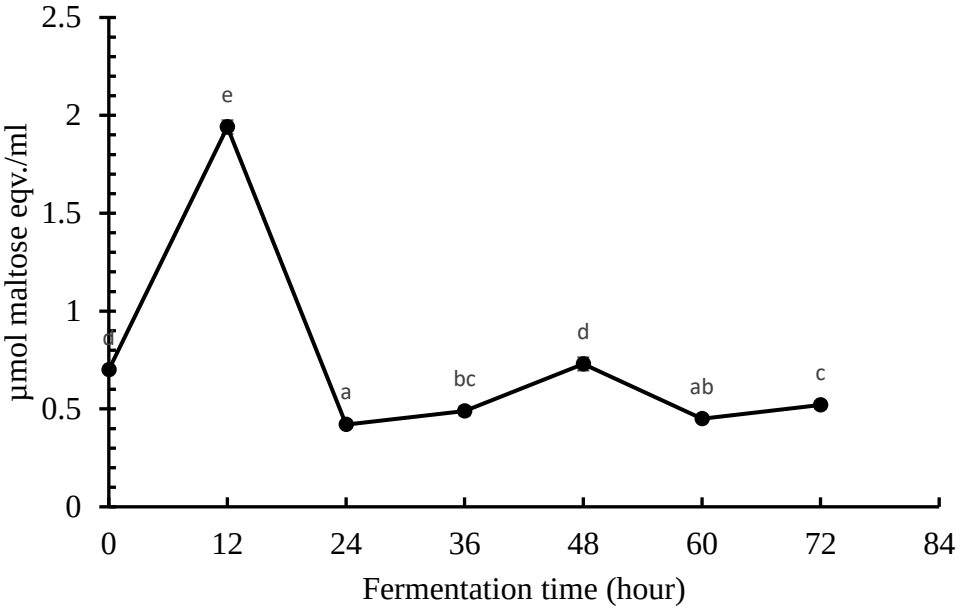
TCA-N increased continuously with the time course of fermentation of black beans by *B. natto* and generally preceded the observed change in ammonia nitrogen. A similar observation was made during a study on '*Thau-nao'*, a traditional Thai-fermented soy product, who found that proteinases released by the dominant species in the inoculum, especially *B.subtilis*, play an important role in proteolysis of soy proteins during fermentation. The degradation of protein into water-soluble nitrogen occurs during fermentation of natto (Hu *et al.*, 2010). A study showed TCA-N increased with fermentation time at first and then decreased in case of natto fermentation. After 36 h of fermentation, non protein nitrogen compounds may be utilized by the starter and resulting in TCA-N decrease (Weng and Chen, 2010b). An increase in TCA-soluble peptides and free α-amino acids of fermented soybean was observed during the time course of fermentation. Proteases secreted by B. *subtilis* growing on soybean surface might hydrolyze soy proteins to oligopeptides and amino acids. These products were subsequently converted to γ-polyglutamic acid, a major component of a viscous material on the soybean surface. Furthermore, those compounds possibly contribute to the characteristic taste and flavor of Thua-nao, especially dimethylprazine and tetramethylpyrazine, which are the main pyrazines detected in soybean-based fermented foods (Visessanguan *et al.*, 2005). Similarly, the degradation of protein into water-soluble nitrogen plays an important role in the characteristic flavor of fermented *natto* (Sarkar and Nout, 2014).

4.5 Effects of fermentation time on Amylase activity

The mean value of amylase activity of raw brown soybean was found to be 0.7 ±0.011 µmol maltose eqv./ml which increased rapidly at 12 h and reached 1.937 ±0.035 µmol maltose eqv./ml and declined as fermentation time increased as shown in fig 4. The rapid increase in amylase activity during initial 12h fermentation time might be due to presence of total sugar in fermenting seed.

The utilization of soluble sugar by increasing population of fermenting organism is also responsible for higher amylase activity (Omafuvbe *et al.*, 2000). However, the activity

gradually declined and accounted to 0.418 ±0.016 and 0.490 ±0.023 µmol maltose eqv./ml at 24 h and 36 h respectively. The decrease in amylase activity is due to decrease in total sugar level (Omafuvbe *et al.,* 2000). Furthermore, the value slightly increased to 0.731 ±0.034 µmol maltose eqv./ml at 48 h.



*Values are expressed in mean of triplicate analysis ± S.D.

Fig: 4.5 Effect of fermentation time on amylase activity

The analysis of variance (Appendix E) showed that there was significant difference ($P<0.05$) in amylase activity of raw soybean and *kinema* fermented at different time interval, while there was no significant difference ($P>0.05$) between amylase content in raw soybean and 48 h of fermentation time. Highest amylase activity is shown in 12 h of fermentation time but result given by (Omafuvbe *et al.,* 2000) shows highest amylase activity in 48 h. This may be due to variety of soybean and variation in total sugar level in fermenting seeds (Omafuvbe *et al.,* 2000).

4.6 Effects of fermentation time on reducing sugar

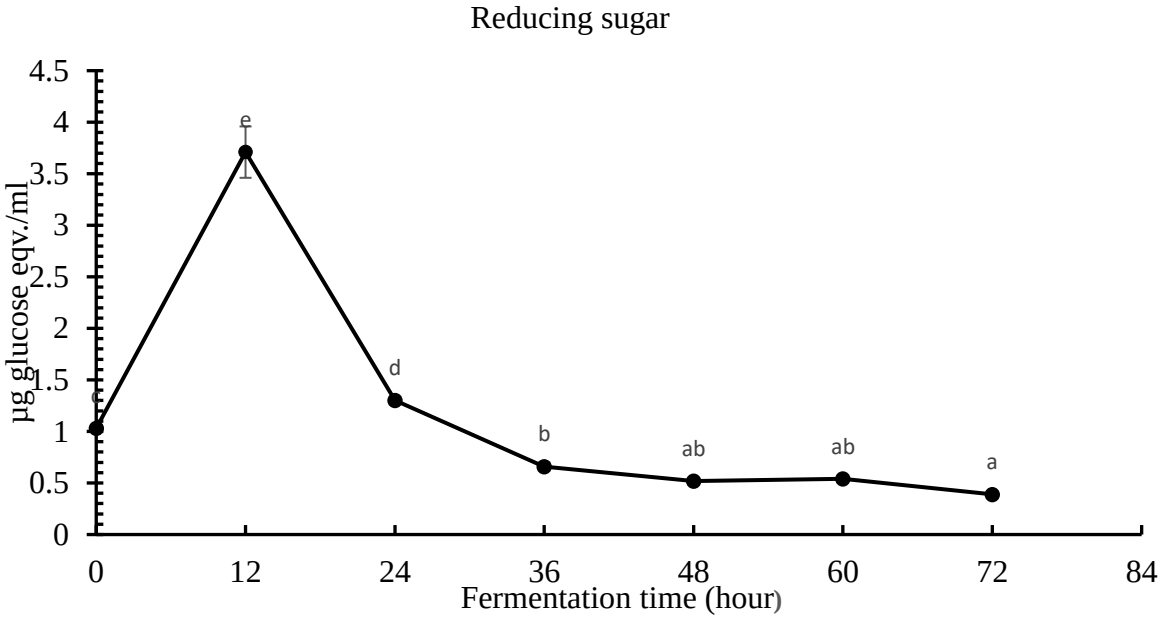
Raw soybeans (0 h fermentation) had a reducing sugar content of 1.033 ± 0.008 µg glucose equivalents/ml. After 12 hours of fermentation, the reducing sugar content peaked at 3.717 ± 0.249 µg glucose equivalents/ml, representing the highest value observed. The total sugar level

is higher in the fermenting seeds causing high amount of reducing sugar in early stage of fermentation (Omafuvbe *et al.*, 2000).

The reducing sugar content started to decline rapidly after the 12-hour peak as shown in fig 4.6.

- 24 h: 1.303 ± 0.038 µg glucose eqv./ml
- 36 h: 0.658 ± 0.040 µg glucose eqv./ml
- 48 h: 0.522 ± 0.027 µg glucose eqv./ml
- 60 h: 0.535 ± 0.028 µg glucose eqv./ml
- 72 h: 0.39 ± 0.015 µg glucose eqv./ml

The analysis of variance (ANOVA) indicated a significant difference ($P<0.05$) between the reducing sugar content of the raw soybean at 0 hours and the content observed at different stages of fermentation.



*Values are expressed in mean of triplicate analysis ± S.D.

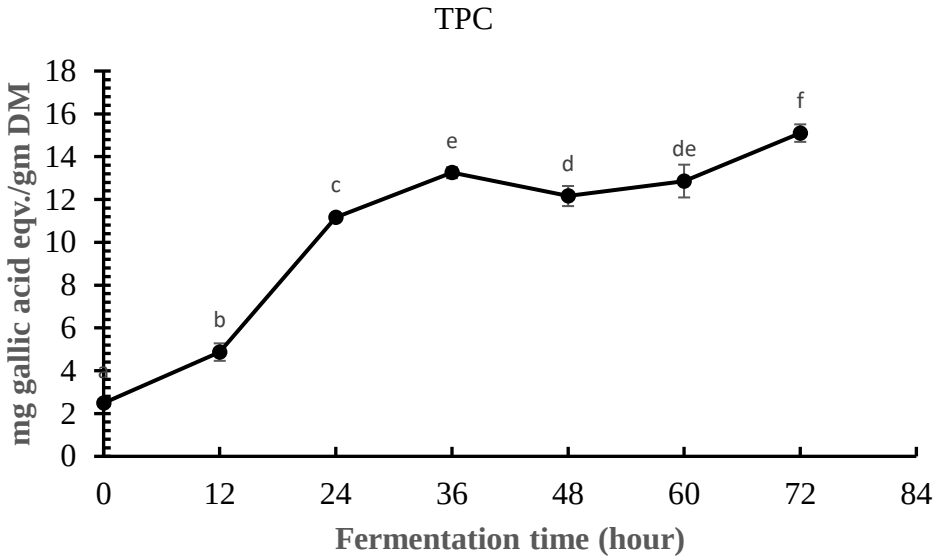
Fig: 4.6 Effect of fermentation time on reducing sugar

The steady decline in total sugar levels during fermentation is likely due to the increasing population of fermenting microorganisms. These organisms utilize the available soluble sugars

as nutrients for growth and reproduction, causing the sugar levels to decrease. As the fermentation progresses, the population of fermenting microorganisms stabilizes. This stabilization likely results in a more constant consumption of sugars, which explains why the soluble sugar levels remain fairly steady in the later stages of fermentation. The enzyme α-amylase breaks down starches into simpler sugars, leading to the initial rise in sugar content during the early hours of fermentation. The subsequent decline in reducing sugar levels is presumably because these sugars are consumed by the fermenting organisms as a source of carbon and energy, which aligns with observations from the study by (Omafuvbe *et al.*, 2000).

4.7 Effects of fermentation time on total phenolic content

The total phenolic component was observed to gradually increase with increase in fermentation time as shown in fig 4.7. The highest value of total phenolic content reached highest at 72h 15.098 ± 0.409 mg gallic acid eqv./ gm DM during 3 days of kinema fermentation. The total phenolic content nearly doubled at 12 h fermentation time as compared to raw soybean. Similar study was shown by (Katuwal *et al.*, 2023) and (Sanjukta *et. al*., 2021).



*Values are expressed in mean of triplicate analysis ± S.D.

Fig: 4.7 Effect of fermentation time on total phenolic content

Similarly, the values remained nearly constant at 48 h and 60 h. The analysis of variance (Appendix E) showed that there was significant difference ($P<0.05$) between raw soybean and fermented *kinema* at different time interval. *Kinema* had significantly higher contents of total

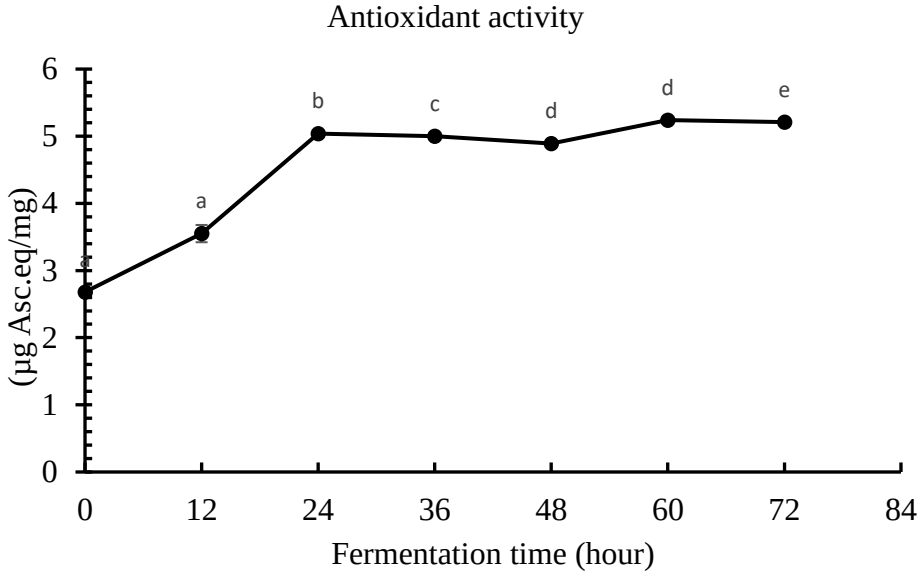
phenolics and flavonoids compared to unfermented soybeans while an increase in fermentation time decreased their contents. The microbial activities could release bound phenolics to the free forms from the soybean resulting in increased contents after fermentation. *kinema* is reported to contain a higher amount of phenolics compared to non-fermented soybean (Katuwal *et al.*, 2023). Similar result was reported in *ting* (fermented sorghum) by (Adebo *et. al*., 2018)

4.8 Effects of fermentation time on DPPH radical scavenging activity

The mean antioxidant activity rapidly increased and reached 5.038 ± 0.027 µg ascorbic eqv/mg dry matter at 24h. However, the value started to decline afterwards where 4.997 ± 0.047 µg ascorbic eqv/mg dry matter and 4.885 ± 0.023 µg ascorbic eqv/mg dry matter was observed at 36 h and 48h respectively as shown in fig 4.8. Similar result was reported by (Cho *et. al.*, 2011), where isoflavone content in fermented *Cheonggukjang* soybean increased during fermentation, peaking at 24 h (387.32 mg/kg) and decrease thereafter (372.28 mg/kg, 372.22 mg/kg and 330.09 mg/kg at 36, 48 and 60 h time frame respectively. Similar pattern was reported by Choi et. al., (2007), where amount of isoflavone in *Doenjang* (Korean fermented soybean) was at peak at 30 days fermentation and declined gradually with increase in fermentation time.

The highest mean antioxidant activity was observed at 60 hours, with a value of 5.24 ± 0.037 µg ascorbic eqv/mg of dry matter, followed by a slight decrease to 5.212 ± 0.004 µg ascorbic eqv/mg of dry matter at 72 hours of fermentation. Statistical analysis shows a significant difference ($p < 0.05$) between the raw sample and the other fermentation time points, which is similar to the study reported by Kim and Yoon (1996). Similar pattern of initial increase followed by decrease in DPPH radical scavenging activity have been shown in *kinema* by (Katuwal *et al.*, 2023). All the antioxidant activities of *kinema* are significantly higher than those of unfermented soybean, this may be due to reducing of hydroperoxide, inactivation of free radicals, or complexing with metal ions. Higher antioxidant property is associated to the presence of phytochemicals, such as isoflavones (Yang *et. al*., 2000). While unfermented soybean shows 36% inhibition of linoleic acid peroxidation, *kinema* exhibits antioxidant activity with 44% inhibition. However, the peroxidation inhibition of kinema extracts declines with time and reaches merely 26% after 72 h. The higher level of antioxidant activity in kinema compared with unfermented soybean could be attributed to the extensive hydrolysis of proteins

by *B. subtilis* (Sanjukta *et. al.*, 2021) and a study showed increase of 58% in the overall content of phytosterols during fermentation (Sarkar and Nout, 2014).



*Values are expressed in mean of triplicate analysis ± S.D.

Fig 4.8 effect of fermentation time on DPPH radical scavenging activity

Phytochemical compositions vary in soybean foods, depending on the varieties and processing techniques, like heating and fermentation. Isoflavone content and its composition may be transformed to other components due high pressure steam treatment during fermentation process. It may also be affected by factors like, varieties genetics and environmental stress. Fermentation increases the amount of isoflavone aglycones due to β-glucosidase activity, while causing to decrease isoflavone glucosides. The loss of isoflavones is associated with leaching into water and thermal degradation during fermentation (Cho *et. al.*, 2011).

Part V

Conclusion and Recommendation

5.1 Conclusions

Based on the overall results from this study the following conclusions are drawn:

i. The study was carried out to determine the fermentation kinetics observed at every 12-hour intervals up to 72 h. Moisture content of brown soybean, crude protein, crude fat, total ash and crude fiber increased after fermentation. However, carbohydrates decreased from 30.15 ± 0.387% to 21 ± 0.812% after 3 days of *kinema* fermentation.

ii. Fermentation of *kinema* for 3 days would be suitable at 30°C temperature since most of the activities that were determined in this study, showed higher values within 60 h of fermentation while some started to decline afterwards.

iii. Proteolysis activity in brown soybean peaked at 60 hours of fermentation, measuring 0.190 ± 0.001 U/ml, and then began to decline until day 3 of fermentation. In contrast, amylase activity was significantly higher at 12 hours of fermentation, reaching 1.937 ± 0.035 µmol maltose equivalent/ml, which was approximately three times greater than that in raw brown soybean.

iv. Antioxidant activity in *kinema* was highest at 60 hours of fermentation, reaching 5.24 ± 0.037 µg ascorbic acid equivalent/mg dry matter, with a slight decline towards the end of fermentation. Meanwhile, total phenolic content increased progressively with fermentation time, peaking at 72 hours with a value of 15.098 ± 0.409 mg gallic acid equivalent/g dry matter.

v. Reducing sugar content, measured as maltose, was highest at 12 hours of *kinema* fermentation, with a concentration of 3.717 ± 0.249 µg glucose equivalent/ml. This value then declined rapidly. On the other hand, TCA-soluble N_2 increased with each 12-hour interval during fermentation, highest at 60 hours with a concentration of 101.382 ± 1.786 µg tyrosine equivalent/ml, before starting to decrease.

vi. The protein content was highest on day 3 of soybean fermentation, measuring 2.758 ± 0.097 mg/ml. During the fermentation process, the pH value increased from 6.74 to 8.5 compared to that of raw brown soybean.

vii. Overall, the study indicated the Protease activity, protein, TCA-N along with release of bioactive constituents were higher during the third day of fermentation, while the amylase activity and the value of reduction sugar inclined during the first day of fermentation.

5.2 Recommendations

Based on this research following recommendation can be made.

i. Changes in cellulase and activity lipase could be done.
ii. Study on fatty acid profile and amino acid profile can be studied.
iii. Fermentation period of 3 days was suitable for functionally and nutritionally adequate *kinema* prepared at temperature of 30°C.

Part VI

Summary

Fermentation, one of the most ancient and economical methods of food preparation in the world, is defined as a technology in which the growth and metabolic activities of microorganisms are used to preserve foods. It is a proven method to improve flavor, texture and nutritional quality of the soybeans. Besides bringing physio-chemical and sensory quality changes, fermentation contributes towards the preservation of food due to release of metabolites that discourage the growth of pathogenic bacteria in foods. Fermentation involves a range of microorganisms such as lactic acid bacteria, acetic acid bacteria, yeasts , molds and a range of bacteria. Similarly, it also covers wide range of products such as staples, adjuncts to staples, condiments and beverages that use substrates such as cereals, pulses, soybeans, flowers, milk, meat etc.

Brown soybeans were purchased from the local market of Dharan, were subjected to preliminary treatment. Soybean was soaked overnight followed by fermentation for 3 days. *Kinema* fermented for different time interval (0, 12, 24, 36, 48, 60 and 72 h) were taken for further analysis.

The pH of kinema fermentation began at 6.8 at 0 hours, dropping to 6 by 12 hours. After this, the pH gradually increased to 7 at 24 hours and reached 8.5 after 72 hours of fermentation. Protease activity peaked at 0.190 ± 0.001 U/ml at 60 hours, with values of 0.181 ± 0.003, 0.190 ± 0.001, and 0.162 ± 0.016 U/ml recorded at 48, 60, and 72 hours, respectively. Amylase activity was highest at 1.937 ± 0.035 µmol maltose eqv./ml at 12 hours and showed fluctuations, with values of 0.418 ± 0.016, 0.490 ± 0.023, 0.731 ± 0.034, 0.452 ± 0.011, and 0.522 ± 0.019 µmol maltose eqv./ml at 24, 36, 48, 60, and 72 hours, respectively. The reducing sugar content spiked at 12 hours, reaching 3.717 ± 0.249 µg glucose eqv/ml, before declining during subsequent time intervals.

The protein content in kinema increased steadily throughout the fermentation process, starting at 0.702 ± 0.066 mg/ml at 0 hours and rising to 2.758 ± 0.097 mg/ml by 72 hours. Intermediate values recorded were 0.831 ± 0.030 mg/ml at 12 hours, 1.339 ± 0.121 mg/ml at 24 hours, 1.589 ± 0.022 mg/ml at 36 hours, 2.242 ± 0.088 mg/ml at 48 hours, and 2.171 ± 0.109 mg/ml at 60 hours. TCA-soluble nitrogen (N_2) also showed a significant rise, starting at 31.291

± 3.575 µg/ml tyrosine at 0 hours and increasing to 101.382 ± 1.786 µg/ml at 60 hours, its highest point. Values for TCA-soluble nitrogen at 12, 24, 36, 48, and 72 hours were 64.952 ± 0.516, 73.345 ± 2.585, 75.333 ± 1.993, 74.6 ± 1.056, and 74.345 ± 0.410 µg/ml tyrosine, respectively.

Total phenolic content increased till 36 h but after a slight decline, the value reaches maximum at 72 h with the value 15.098 ± 0.409 mg gallic eqv./gm DM. The antioxidant activity gradually increased as 2.676 ± 0.007 , 3.550 ± 0.128 and 5.038 ± 0.027 µg Asc. Eqv /mg DM at 0,12 and 24 h. The value slightly decreased at 36 and 48 h and attained the highest activity of 5.240 ± 0.037 µg Asc. Eqv /mg DM at 60 h and the value again dropped at 72 h.

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Appendices

Appendix A

Table A.1 Equipment and utensils

Incubator	Spectrophotometer
Micropipette	Water bath
Mortar and pestle	Measuring cylinder
Weighing balance	Test tubes
Glass rod	Conical flask
Beaker	Filter paper
Centrifuge machine	Pipettes
Magnetic stirrer	Petri-plates
Refrigerator	pH meter
Hot air oven	

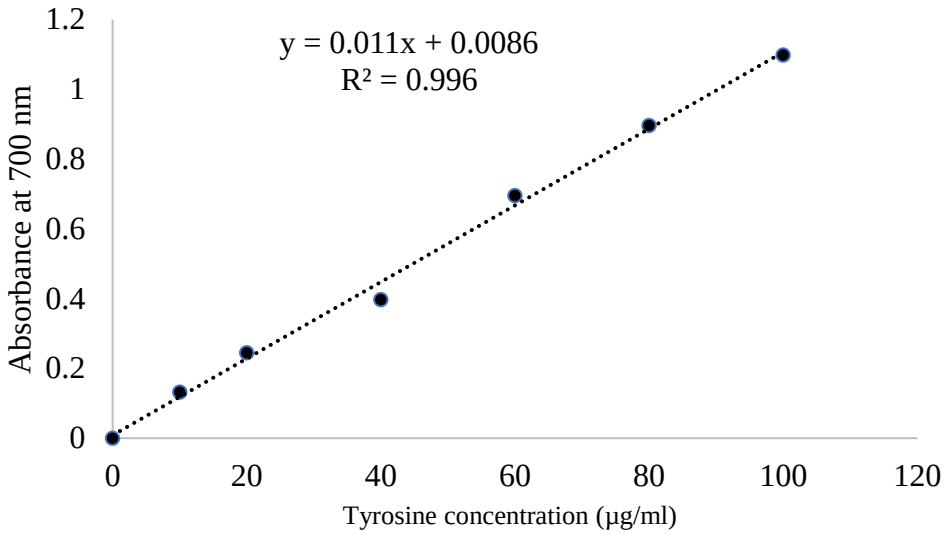
Appendix B

Table B.1 Chemicals used during research work

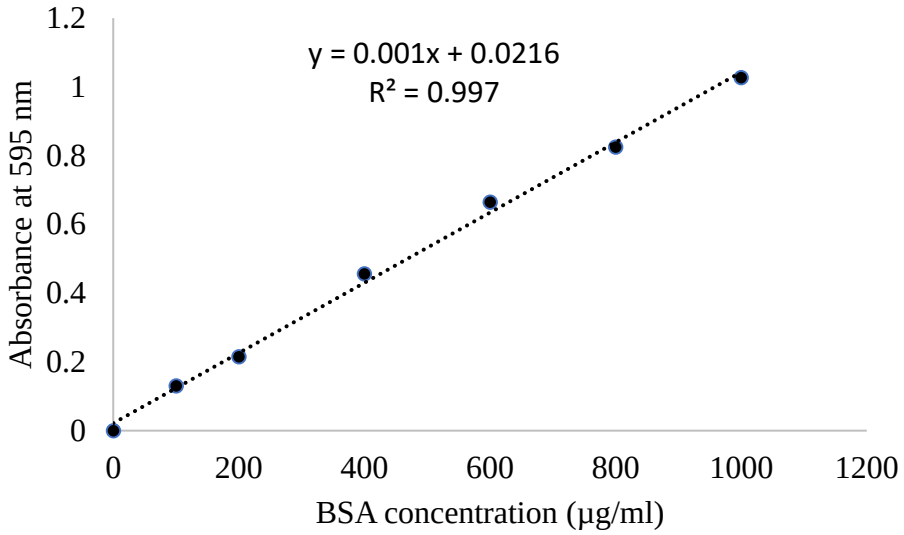
Sodium phosphate dibasic	Sodium potassium tartarate
Sodium dihydrogen phosphate	Sodium Sulphite
Tri-chloroacetic acid	Phenol
Casein	Starch
NaoH	Methanol
Copper sulphate	Sodium Carbonate
Gallic acid	Maltose

Appendix C

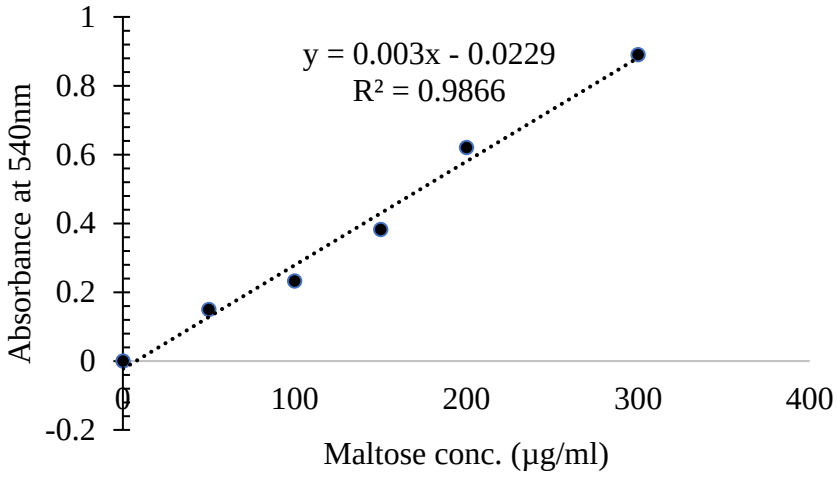
C.1 Standard curve of tyrosine for protease activity assay



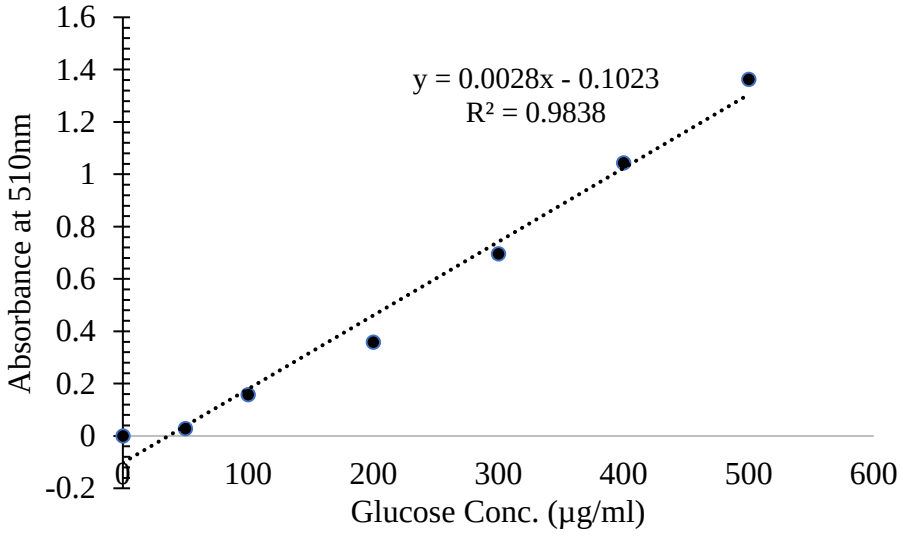
C.2 Standard curve of BSA for protein content determination



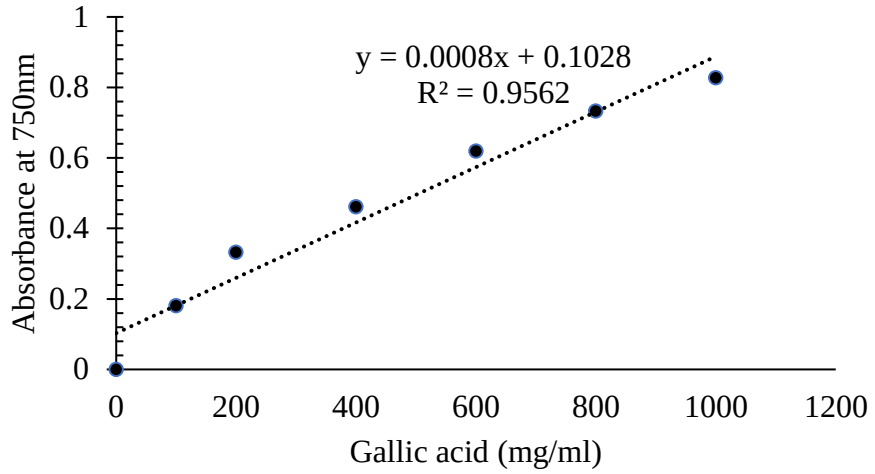
C.3 Standard curve of maltose for Amylase activity determination



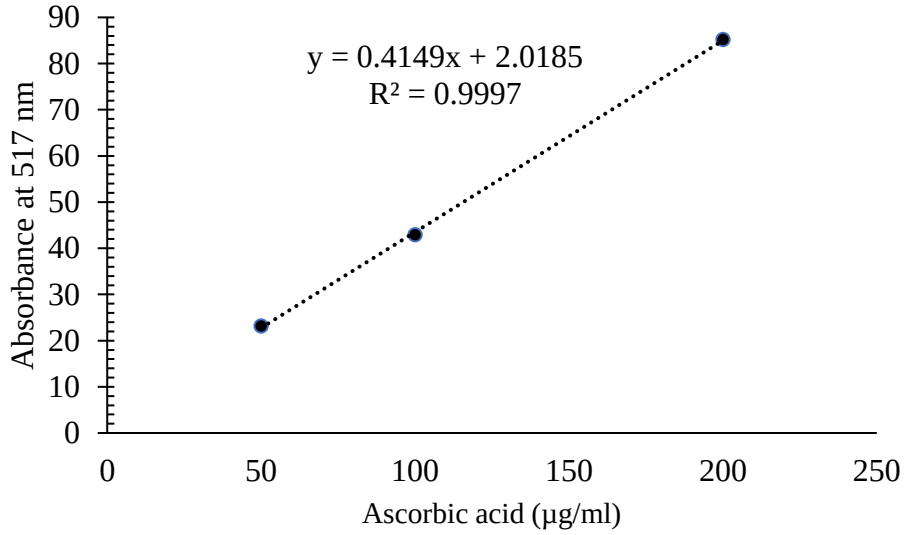
C.4 Standard curve of glucose for reducing sugar determination



C.5 Standard curve of gallic acid for total phenolic content determination



C.6 Standard curve of Ascorbic acid for antioxidant activity



Appendix D

D.1 Preparation of reagents

1. Preparation of Phosphate buffer for pH7

   - Mixing 39.0ml 0.2M NaH2PO4.2H2O and 61.0ml 0.2M Na2HPO4. 2H2O

2. Preparation of Bradford reagent

   - 0.01 g Coomassie Brilliant Blue G-250 is added to 5 ml ethanol (95%) and 10 ml phosphoric acid (85%).
   - Volume make up is done with distilled water to 100 ml.

3. Preparation of Bovine serum albumin (BSA)

   - 0.2 g BSA is added to 100 ml distilled water.

4. Preparation of DNSA reagent

   - 3ml of DNS reagent and add 1 ml of 40% Rochelle salt solution

Appendix E

Table E.1 ANOVA for protease activity

Samples	Mean	Significance	l.s.d	d.f
0	-0.008	a	0.01260	14
12	-0.006	a		
24	0.055	b		
36	0.139	c		
48	0.181	e		
60	0.190	e		
72	0.162	d		

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	**F .pr.**
Treatment	6	0.159166	0.026528	16.75	<.001
Residual	14	0.022172	0.001584		
Total	20	0.181338			

Table E.2 One way ANOVA at 5% level of significance for Amylase activity

Samples	Mean	Significance	l.s.d	d.f
0	0.7003	d	0.04050	14
12	1.9371	e		
24	0.4176	a		
36	0.4898	bc		
48	0.7307	d		
60	0.4522	ab		
72	0.5221	c		

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	**F .pr.**
Treatment	6	5.1925964	0.8654327	1617.72	<.001
Residual	14	0.0074896	0.0005350		
Total	20	5.2000860			

Table E.3 One way ANOVA at 5% level of significance for Antioxidant activity

Samples	Mean	Significance	l.s.d	d.f
0	0.702	a	0.1472	14
12	0.831	a		
24	1.339	b		
36	1.589	c		
48	2.242	d		
60	2.171	d		
72	2.758	e		

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	**F .pr.**
Samples	6	10.556967	1.759495	248.91	<.001
Residual	14	0.098963	0.007069		
Total	20	10.655931			

Table E.4 One way ANOVA at 5% level of significance for protein

Treatment	Mean	Column A	l.s.d	d.f
0	0.702	a	0.1472	14
12	0.831	a		
24	1.339	b		
36	1.589	c		
48	2.242	d		
60	2.171	d		
72	2.758	e		

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	**F .pr.**
Samples	6	10.556967	1.759495	248.91	<.001
Residual	14	0.098963	0.007069		
Total	20	10.655931			

Table E.5 One way ANOVA at 5% level of significance for Reducing Sugar

Samples	Mean	Column A	l.s.d	d.f
0	1.033	c	0.1714	14
12	3.717	e		
24	1.303	d		
36	0.658	b		
48	0.522	ab		
60	0.535	ab		
72	0.390	a		

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	**F .pr.**
Treatment	6	24.656738	4.109456	429.12	<.001
Residual	14	0.134069	0.009576		
Total	20	24.790807			

Table E.6 One way ANOVA at 5% level of significance for TCA soluble N_2

Treatment	Mean	Significance	l.s.d	d.f
0	31.29	a	3.513	14
12	64.95	b		
24	73.35	c		
36	75.33	c		
48	74.60	c		
60	101.38	d		
72	74.35	c		

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	**F .pr.**
Treatment	6	7753.331	1292.222	321.05	<.001
Residual	14	56.350	4.025		
Total	20	7809.681			

Table E.7 One way ANOVA at 5% level of significance for TPC

Treatment	Mean	Significance	l.s.d	d.f
0	2.493	a	0.7315	14
12	4.866	b		
24	11.169	c		
36	13.245	e		
48	12.161	d		
60	12.858	de		
72	15.098	f		

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	**F .pr.**
Treatment	6	398.7922	66.4665	380.89	<.001
Residual	14	2.4431	0.1745		
Total	20	401.2422			

Appendix F

Plates

Plate 1 Incubation of Kinema

Plate 2 Kinema after 3 days of fermentation

Plate 3 Samples of 12 h differences stored in refrigeration

Plate 4 Sample for DPPH analysis

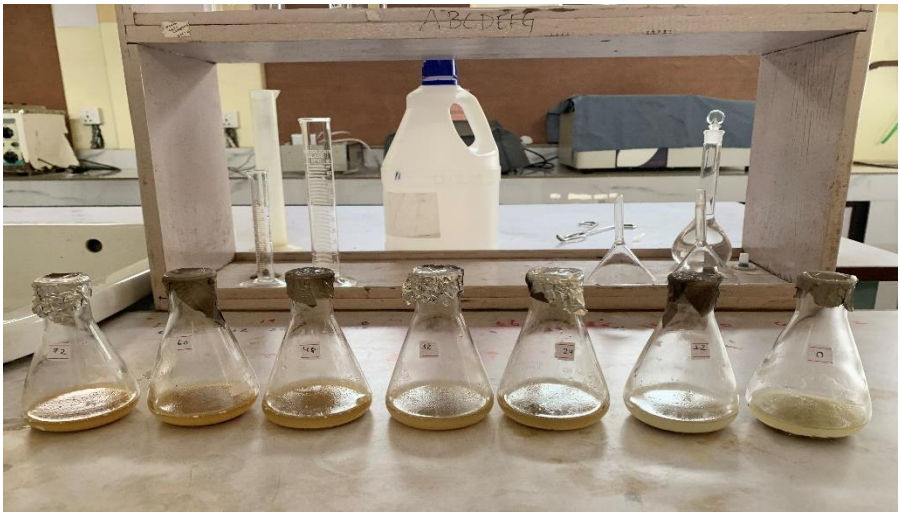


Plate 5 Methanol extracts of sample for antioxidant and TPC determination

Plate 6 DNS ready sample obtained from shaker

Plate 7 Collection of sample after hydrolysis by TCA for protease activity