EXTRACTION OF PAPAIN ENZYME FROM PAPAYA LEAVES USING HOT WATER EXTRACTION WITH ULTRASONIC – ASSISTED PRETREATMENT

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ABSTRACT

Papaya leaf is not just a byproduct of papaya tree but it contains an essential enzyme which is papain. The aim of the study is to measure the amount of this enzyme extracted from leaf tissue using hot water extraction with ultrasonic-assisted pretreatment as well as to determine the optimum condition for the extraction process. The leaves were subjected to a pretreatment which is grinding and ultrasonication followed by hot water extraction and finally, enzymatic analysis using UV-Vis spectrophotometer. The parameters involved in the process were ultrasonication time (30 – 90 minutes) and frequency (18 – 26 kHz), solid-solvent ratio (1:5 – 1:25), and extraction time (1 – 5 hour) and temperature (50 - 70°C). Among these parameters, maximum concentration of papain enzyme was achieved at 60 minutes and 20 kHz of ultrasonication, solid-solvent ratio at 1:5, extraction time at 4 hours and extraction temperature at 60°C. In conclusion, the maximum concentration of papain enzyme achieved at this optimum parameter was 4.020 µM. Utilization of PEG–phosphate system for separation and purification of papain is recommended in order to get the pure papain enzyme.

ABSTRAK

Daun betik bukan sahaja dilihat sebagai hasil sampingan daripada pokok betik malah ia juga mengandugi enzim penting iaitu papain. Tujuan kajian ini adalah untuk mendapatkan kandungan papain yang diekstrak daripada tisu daun menggunakan kaedah pengekstrakan air panas dengan pendekatan pra-rawatan ultrasonik begitu juga dengan mendapatkan parameter yang paling optimum untuk preoses ekstraksi**.** Daun betik akan melalui kaedah pra-rawatan iaitu pengisaran dan ultrasonik diikuti dengan pengekstrakan menggunakan air panas dan akhir sekali ialah enzim analisis menggunakan Ultraungu Spektrofotometer**.** Parameter yang terlibat dalam proses ini ialah tempoh ultrasonik (30 – 90 minit) dan frekuensi (18 – 26 kHz), nisbah pepejal-pelarut (1:5 – 1:25), masa pengekstrakan (1 – 5) dan suhu (50 – 70 °C). Di kalangan parameter – parameter tersebut, kepekatan enzim papain yang paling tinggi telah dicapai pada minit ke 60 dan proses ultrasonik pada 20 kHz, nisbah pepejal-pelarut pada 1:5, masa pengekstrakan selama 4 jam, and suhu pengekstrakan pada 60°C. Kesimpulannya, kepekatan enzim papain yang paling tinggi yang telah dicapai pada parameter yang optimum ialah 4.020 µM. Penggunaan sistem PEG- fosfat untuk pengasingan dan penulenan adalah disarankan untuk mendapatkan enzim papain yang tulen.

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LIST OF SYMBOLS

Kg	-	kilogram
g	-	gram
%	-	percentage
°C	-	degree Celsius
hr	-	hour
kHz	-	kilohertz
Da	-	Dalton
mL	-	milliliter
mm	-	millimeter
µm	-	micrometer
HPLC	-	High Performance Liquid Chromatography
min	-	minute
w/v	-	weight per volume
v/v	-	volume per volume
g/l	-	gram per liter
mol/l	-	mol per liter
mM	-	milimolar
cm	-	centimeter
rpm	-	revolution per minute
nm	-	nanometer
L	-	Liter
V	-	Volt
W	-	Walt

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CHAPTER 1

INTRODUCTION

1.1 Background of study

The papaya plant is a small tree which is from Caricaceae family. It is originated from the lowlands of eastern Central America, from Mexico to Panama. Papayas have many common names such as pawpaw tree, papaya, papayer, tinti, pepol, chich put, fan kua, and kepaya. Papaya is a large tree-like plant with a single stem growing from 8 to 10 metres tall. Papaya leaves are large, 45–70 centimetres diameter, deeply palmately lobed with 7 lobes. The fruit is ripe when its skin has attained amber to orange hue and has bitter sweet taste. Its weight usually is around 2.5kg.

Figure 1.1: Papaya tree

The leaves, seeds, skin and fruit of the papaya contains a few enzymes. Main enzymes are papain, chymopapain, pectin, carposide, carpaine, pseudocarpaine, dehydrocarpines, carotenoids, cryptoglavine and antheraxanthin. Papain enzyme which is sometimes called as vegetable pepsin is the cysteine protease enzyme which consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group and it is necessary for activity of the enzyme.

Papain is the enzyme used in commercial meat tenderizers. It also has the ability to dissolve dead tissues without damaging the cells (Mahmood, Sidik & Salmah, 2005). Papain has many uses and functions in a variety of industries: clarifying beer, meat tenderization, clarifying preservation of spices, contact lens cleaners, detergents, pet food palatability, digestive aids, blood stain remover, blood coagulant and cosmetics. Papain is also widely used for many medical and para-medical purposes such as to assist protein digestion in chronic dyspepsia, gastric fermentation, gastritis, removal of necrotic tissue, preparation of tyrosine derivatives for the treatment of Parkinsonism, preparation of tetanus vaccines, skin cleansing agents, acne treatment.(Choudhury et al.,2009) The demand for more stable, highly active and specific enzymes is growing rapidly (Bhat, 2000).

Papain enzyme usually obtained by doing the extraction process. Extraction is a process of obtaining something from a mixture or compound by chemical or physical or mechanical means. There are several of extractions methods can be used such as Subcritical Water Extraction (SCWE), Microwave-Assisted Extraction (MAE) method, Soxhlet extraction, Supercritical Fluid Extraction (SCFE), Pressurized Solvent Extraction (PSE) and Hot Water Extraction (HWE).

Hot water extraction is an extraction method which is easy to be applied by industry without requiring expensive extraction equipment. In the extraction of papaya leaves by Anibijuwon and Udeze (2009), 25grams of the finely ground leaves were suspended in 125milliliter of distilled water or 95% ethanol or methanol. The hot water extraction was done at 80°C in a water bath for 1/2hours while the ethanolic and methanolic extraction was done at 28°C for 120 hours. The extracts were then filtered through a filter paper and were then stored in the refrigerator at 4°C until used.

Pre-treatment is the first process before going on with extraction process. The material needs to be homogenized or ground into a cellular suspension and the cells are then subjected to cell disruption to release the product into a solution. The intracellular of cell can be disrupted by various techniques, one of them is ultrasonication.

There are two types of ultrasonic which are ultrasonic probe system and ultrasonic cleaning bath. High frequency vibrations that applied during ultrasonication cause the formation of tiny bubbles within the liquid medium. When these bubbles reach resonance size, they collapse releasing mechanical energy in the form of shock waves. This shock wave disrupts the cell present in the suspension. A frequency of 25 kHz is commonly used for cell disruption while duration of ultrasonic depends on the cell type, sample size and the cell concentration.

1.2 Problem Statement

Papain is commonly extracted from the latex. The latex of the papaya is obtained by making an incision on unripe green fruit (Thomas et al., 2009). This incision would leave scars on the fruit resulting in lower market value. Instead of using fruit, leaves also contain papain enzyme and utilization of papaya leaves can reduce the amount of waste.

Organic solvent is one of an extracting agent used to extract papain enzyme. The utilization of this organic solvent such as methanol, benzene and acetone in the extraction process will need further process in order to purify the extraction yield. Besides, these organics solvent quite harmful for human and their utilization in extraction process should be minimized. It is better and safe to use green solvent in order to extract the papain enzyme.

1.3 Objectives of Study

The objectives of study are measuring the amount of papain extracted from leaf tissue using hot water extraction with ultrasonic assisted pretreatment and to determine the optimum condition for the extraction process.

1.4 Scope of Study

In order to achieve the objectives of the study, the following scopes have been identified. The effect of papain pre-treatment conditions should be identified which is ultrasonication and solid-solvent ratio. The effect of cell disruption using ultrasonic that would be identified is duration for ultrasonic and its frequency. The duration time for ultrasonication process would be varied from 30 to 90 minutes and its frequency is 18 to 26 kHz. Next is solid-solvent ratio which would be varied from 1:5 to 1:25. Last scope is the effect of hot water extraction's temperature and time which is 50°C,55°C 60°C,65°C and 70°C for 1, 2, 3, 4 and 5 hours.

1.5 Rationale and Significance

It is known that papaya leaves is one of by-products from papaya tree. These by-products are generally disposed in open areas. Rather than that, one potential alternative use for these wastes is extraction of proteolytic enzymes such as papain and other cysteine proteases which produce valuable products. Utilization of leaves can create waste to wealth, hence reducing the disposal problem.

Hot water extraction is one of low cost method used to extract papain but it is not promised to get high concentration of papain. In this research, pretreatment is proposed to improve the efficiency of the extraction method, hence increasing the yield of papain.

Furthermore, it has been proved that papain enzyme from papaya leaves can gives benefit to human health. This papain can be used as remedy against various disorders, including cancer and infectious diseases. Thus, this papaya leaves can create waste to health as it have a valuable enzyme that can be utilized as medical product.

CHAPTER 2

LITERATURE REVIEW

2.1 Papaya

2.1.1 Origin

The papaya plant is from Caricaceae family, originated from the lowlands of eastern Central America. Its seeds, which remain viable for several years if dried, were distributed to the Caribbean and south-east Asia (Philippines) during Spanish exploration in the 16^{th} Century, from where it was further distributed to India, the Pacific and Africa. Papaya was introduced into Hawaii in the early 1800s by the Spanish explorer Don Francisco Marin and became an export crop of Hawaii in 1948. Today, papaya is widely distributed throughout the tropical and warmer subtropical areas of the world and has become naturalized in many areas (Department of Health and Ageing, 2008).

2.1.2 Morphology

Papayas have many common names such as pawpaw tree, papaya, papayer, tinti, pepol, chich put, fan kua, and kepaya. Other names associated with papaya include tepayas by Kadazan Dusun community in East Malaysia, betik in Peninsular Malaysia, lechosa in Venezuela, pawpaw in Sri Lanka and papali in India. It is a polygamous diploid plant species with three sex types which is male, female and hermaphrodite on the same plant (Urasaki et al., 2002).

Papaya is a short-lived, fast growing, large tree-like plant with a single stem growing from 8 to 10 metres tall, with spirally arranged leaves only at the top of the trunk. Papaya leaves are large, 45–70 centimetres diameter, deeply palmately lobed with 7 lobes. The flowers are wax-like. Male flowers are in clusters while female are sessile. They appear on the axils of the leaves, maturing into the large 15–50 centimetres long, 10–30 centimetres diameter fruit. The life of leaves is between 2.5 to 8 months and new leaves arise at the rate of 1.5 to 4 per week. The fruits are classified as freshy berries since the resemble melon by having a central seed cavity. They are born axis to the main stem in small cluster.The fruit is ripe when its skin has attained amber to orange hue and has bitter sweet taste. Its weight usually is around 2.5kg.

Papaya is extremely sensitive to frost, and water-logging will kill the taproot within forty-eight hours. The Papaya is also susceptible to parasites, pests and diseases. This fussy plant needs a lot of water but must have good drainage, and it bears most fruit in light, porous, slightly acidic soils that are rich in organic matter.

2.1.3 Advantages

Papaya has so many advantages. Papaya seed is found to be a rich source of biologically active isothiocyanate. Unripe pulp of C. papaya is rich in carbohydrate and starch and also contains cardenolides and saponins that have medicinal value such as cardenolides used in the treatment of congestive heart failure. Papaya latex showed marked in vivo efficacy against the rodent gastrointestinal nematode, Heligmosomoides polygyrus. The seeds are also used as emmenagogue, thirst quenchers, carminatives or for bites and stings of poisonous insects. In Cambodia, Laos and Vietnam, latex is used to treat eczema and psoriasis. Carpaine, an alkaloid with an intensely bitter taste and a strong depressant action on the heart, has been obtained mainly from the leaves, fruit and seeds. In Kelantan (a state of Malaysia), the latex of the unripe fruit is used as a poison for criminal purposes. The Kadazan Dusun community in Malaysia uses the decoction of papaya root as a means of birth control, preventing menstruation and for uterine contractions after birth (Anuar et. al, 2003). Two enzymes from papaya that have very strong digestive properties are papain and chymopapain (Mahmood, Sidik & Salmah, 2005). Papaya has many uses, including as food, as cooking aid and as medicine.

Papaya fruit is highly appreciated worldwide for its flavor, nutritional qualities and digestive properties. Papaya has many uses, including as food, as cooking aid and as medicine. The leaves, seeds, skin and fruit of the papaya contain an essential enzyme that can restore and rejuvenate the skin naturally so that you look and feel great. The stem and the bark are also used in rope production (Anibijuwon and Udeze, 2009). The papaya is rich in many nutrients. These nutrients include vitamin E, potassium, fiber, vitamin A, vitamin C and vitamin K.

2.2 Enzyme

Enzymes are extremely versatile compounds that can increases the rate of a wide range of chemical reactions. Because of their characteristics, they are, in principle, ideal catalysts for use in analytical applications, as they act in aqueous solutions at ambient temperatures and atmospheric pressure. Most of them are proteins. Almost all processes in a biological cell need enzymes in order to react at significant rates. Enzymes usually work by lowering the activation energy for a reaction, hence increasing the rate of the reaction. Mostly, enzyme reaction rates are millions of times faster compared to un-catalyzed reactions.

For most enzymes, their activity is temperature dependant until a maximum is reached, when the enzyme is no longer stable; also, like other proteins, enzymes are stable over only a limited range of pH. Outside this range, changes in the charges of ionizable amino-acid residues result in modifications to the tertiary structure of the protein and eventually lead to enzyme denaturation and inactivation (Bermejo et al., 2004). The enzymes are commonly used in many industrial applications. The demand for more stable, highly active and specific enzymes is growing rapidly (Bhat, 2000).

2.2.1 Papain

Papain enzyme comes from papaya which is sometimes called as vegetable pepsin. Papain is the cysteine protease enzyme which consists of a single peptide chain of 211 amino acids. These amino acids are strung together in a chain which is folded due to the presence of disulfide bridges. Papain molecules have molecular weight of 23,000 Da and an isoelectric point of 9.5. Papain preferably cleaves peptide bonds involving basic amino acids and it also has an esterase activity. The catalytic site of the enzyme contains a catalytic triad at the 25th position, histidine at the 159th position and asparagine at the 158th position, which exists as zwitterions. (Sangeetha, Abraham, 2006). These three amino acids are in close proximity due to the folding structure and they are working together in the active sites that provide this enzyme with its unique functions.

Papain contains a sulfhydryl group at the active site, which is the portion of the enzyme that contains the reaction and binding sites. This active site is located at the 25th amino acid and creates a small loop in the molecule involving the next approximately 30 amino acids. The positioning of the active site not only makes papain unique, but also makes it useful for studying other similar proteolytic enzymes. The remaining sulfides of the enzyme are found on three distinct disulfide bridges. These bridges are important to the structure because they require that the molecule must be folded, therefore creating a three dimensional structure. The first bridge occurs between amino acid 22 and 159 which is important as this brings the three amino acids of the active side together. In addition, the way the molecule is folded along these bridges created a strong interaction among the side chains which contributes to the stability of the enzyme.

2.2.2 Papain from Leaf

Papaya leaves have been shown to contain many active components such as papain, chymopapain, cystatin, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates (Seigler et al., 2002).

Analysis show that leaves, petioles and stalks of papaya also contain papain. It is also stated that in the earlier study, the methods of obtaining leaf papain are chemically possible. Proteolytic study of the enzymatic extracts from leaf shown that leaf tissue exhibit the highest overall extract yield, extract protein content and total yield. Besides, leaves of papaya can be obtained throughout the years of cultivation and the quality only differs in the quantity of juice acquired (Balls & Thompson, 1940).

2.2.3 Papain from Latex

Papain was the first cysteine proteinase to be characterized as a latex component in Carica papaya, and became a protein model in structural and kinetic studies for cysteine proteinases. The relatively easy of producing milligram amounts of this enzyme stems from its high concentration amounting to about 1% of the total protein in papaya fruit latex before ripening, thus providing another example for the temporal expression of these enzymes. Papain is found in latex along with five isoforms of chymopapains, proteinase omega, and proteinase IV. It is likely that other short lived proteinases also occur in latex, but their identity has not yet been confirmed in latex preparations, obtained by tapping the plant fruit by non-rigorous collection protocols (Salas et al., 2008).

Papain is usually extracted from the latex of the green fruit. It is reported that the latex of the green papaya contains much papain and higher enzymatic activity (Balls & Thompson, 1940). Classic method of extracting papain from the papaya involve the collection of latex by making incision on the unripe fruit and further processing of drying the latex will result in papain powder. However, the effect of making incision on unripe green fruit would leave scars on the fruit resulting in lower market value.

The papaya-latex is well known for being a rich source of the four cysteine endopeptidases namely papain, chymopapain, glycyl endopeptidase and caricain (Azarkan et al., 2003) and the content may vary in fruit, leaves and roots. Commercially, papaya latex is harvested from fully-grown but unripe fruit. Ripe papaya contains less latex compared to green papaya possibly due to cessation of function or breakdown with age of the latexproducing cells (OECD, 2005).

2.2.4 Benefits of Papain

Many scientific investigations have been conducted in order to evaluate the biological activities of various parts of papaya, including fruits, shoots, leaves, stalks, seeds, roots or latex. Papaya leaf extract may potentially provide the means for the treatment and prevention of selected human diseases such as cancer, various allergic disorders, and may also serve as immunoadjuvant for vaccine therapy (Noriko et al., 2010).

Similar findings support the antitumoral action of papain from C. papaya. The growth rate, invasiveness and metastasis of both the B16 melanoma and Lewis lung carcinoma were inhibited in mice immunized with papain, increasing the mean survival of treated mice relative to the tumor-bearing untreated controls. The protection is mediated by serum antibodies cross-reacting with cathepsin-B- and cathepsin-H-like endopeptidases isolated from B16 melanoma cells (Bellelli et al., 1990), as seen for fastuosain. Further studies showed that long-term rectal administration of an enzyme mixture containing papain, trypsin and chymotrypsin displayed antitumoral effect in C57Bl6 inbred mice inoculated with Bl6 melanoma cells (Wald et al., 1998). In addition, the growth inhibition of primary tumors and metastasis was correlated with decreased expression of CD44 and CD54 molecules in animals exposed to proteolytic enzymes, suggesting that serine and/or cysteine proteinases suppress B16 melanoma, and restrict metastatic dissemination in C57B16 mice (Wald et al., 2001). Our group has shown that in melanoma murine models P1G10 from C. candamarcensis reduces the tumor size and the frequency and number of lung metastases, the latter by about 75%, with a significant increase in survival (Figueiredo et al., unpublished data). Taken together these findings clearly show the beneficial effects of plant cysteine proteases as principles endowed with antitumoral and antimetastatic effects (Salas et al.,2008).

This review focuses on some pharmacological effects attributed to cysteine proteinases, ignoring other therapeutical options. For instance, the efficacy and safety of cysteine proteinases was established in enzymatic debridements of burns (Rosenberg et al., 2004) and we observed a 25–30% increase in the epithelization rate following periodical applications of P1G10 proteases in rodent subjected to dorsal burns induced by dry-heat (Latini et al., unpublished data). In these experiments the pharmacologically effective dose was about 0.1%, which is equivalent to the dose used for dermal healing. A different approach has been used when using cysteine proteinases as debrider as an alternative to mechanical cleansing for rapid removal of dead tissue. In these protocols the pharmacological concentration of proteases ranges between 2% and 5% (Rosenberg et al., 2004). A number of reports attribute cartilage remodeling and anthirheumatic properties to cysteine proteinases, the latter effect probably related to the immunomodulatory actions attributed to these enzymes (Salas et al., 2008)..

Many scientific investigations have been conducted in order to evaluate the biological activities of various parts of papaya, including fruits, shoots, leaves, stalks, seeds, roots or latex. Papaya leaf extract may potentially provide the means for the treatment and prevention of selected human diseases such as cancer, various allergic disorders, and may also serve as immunoadjuvant for vaccine therapy (Noriko et al., 2010).

Proteases are one of the most important industrial enzymes, accounting for nearly 60% of totalworldwide enzyme sales. Proteases are mainly incorporated in the clothwashing detergents to remove the protein strains of blood, egg yolk and chocolate. The use of protease enzyme in washing clothes is that it hydrolyses the protein strains in the fabric into peptides. But when used in detergents, it requires stability at alkaline pH values and also at low temperatures. (Sangeetha & Abraham,2006).

Since papain has such a variety of uses, it is desirable to obtain this protein in substantial quantities for both basic research and industrial use. It can be used as an alternative to subtilisin, provided that it is stable in harsh conditions and is active at low and high temperatures. Papain is less expensive than microbial enzymes, and has got a wide range of specificity and good thermal stability amongst other proteases. Therefore, it has got high potential to use in detergents. (Sangeetha & Abraham,2006).

2.2.5 Market Overview

Enzyme is in great demand in the international market essentially U.K., U.S.A., Europe and Gulf Countries. The present exporting counties are Zaria, Tanzania, Philllippines, Srilanka, India and Cameroon. Papaya is available in Sabah and other parts of Malaysia, therefore it is highly profitable to manufacture papain where the fruit production is in abundance possible as it is a tropical region. Besides, papain is less expensive than microbial enzymes, and has got a wide range of specificity and good thermal stability amongst other proteases (Sangeetha & Abraham,2006).

The production of papaya fruits in Malaysia is increase to 6.2 percent annually from one million tones in 1996 to 1.4 million tones in 2000 while harvested area grew by 4 percent per annum from 244,500 hectares in 1996 to 297,400 hectares in 2000. In 1992, quantity of 93,930 tonnes of papaya was produced in Malaysia and 23,225 tonnes of papaya were exported to foreign countries Singapore, Hong Kong and Western Europe (Krishnaiah et al, 2002).

In 2006, Mexico produced 798,589 tons of papaya and was the principal papaya exporter to the United States, accounting for 65% of imports. This same year the state of Yucatan, Mexico produced 54,497 tons of papaya, making it the fourth largest producer nationwide (Thomas et.al, 2009).

The food and pharmaceutical enzyme market shows a good expansion potential in the future. The enzyme market is growing at 3% to5% annually. The market potential in the Europe and U.S.A is in great demand. Preliminary analysis of income for the cultivator shows that in one acre of papaya plantation, an amount RM 40,000 revenue could be obtained.

2.3 Pretreatment

Pre-treatment is the first process before going on with extraction process. The pretreatment process need to be done to break the cell wall so that the release of intracellular contains is increase.

There are few methods for this pre-treatment. The papaya fresh leaves were cut and wash with distilled water. Then it would be dried in oven at 50°C for 5-7 days. The leaves were ground using a grinder. Then 50g of blended plant were weighted and placed into 100mL flask. The water was added in ratio 1:20 (Mahmood, Sidik & Salmah, 2005).

Besides, the pre-treatment also can be done by collecting the fresh leaves of Epimedium sagittatum Maxim and immediately cleaned and cut into pieces (3 mm×3 mm). Then the extracting process will be continued (Zhang, Yang, Zhao, and Wang, 2009).

2.4 Cell Disruption

2.4.1 Grinding

Sayyar et al., (2009) observed that the particle size of sample is one of the factors affecting the extraction process. Less oil is extracted when larger particleis applied compared to the small size of particles.

The plant particle size and the status in which it is presented for MAE can have a profound effect on the recoveries of the compounds. The particle sizes of the extracted materials are generally in the range of 100 μm – 2 mm. Fine powders can enhance the extraction by providing larger surface area, which provides better contact between the plant matrix, and the solvent, also finer particles will allow improved or much deeper penetration of the microwave (Mandal, Mohan & Hemalatha, 2007).

Zayas, (1986) also get the same effect where exraction of Chymosin give the high yield when shredding the Chymosin into strips of 5 to 8 mm width compare to cutting it into 150 x 150 mm.

Three of these research stressed out that small size of sample will enhance the extraction process. Thus, grinding process is applied in this research as a pretreatment process.

2.4.2 Ultrasonication

The use of ultrasound to enhance the extraction yield is a technique that started in the 1950s with laboratory scale experiments (Vinatoru, 2009). The main mechanism of ultrasound-assisted extraction is the disruptions of leaf tissues and cell walls, which result from cavitation bubbles collapse, a phenomenon occurring in liquid medium under the impact of ultrasound, thus improving the mass transfer of the solvents into the leaf materials and the soluble constituents into the solvents (Zhang et al. 2009).

There are several types of ultrasonic equipment. All of them are shows as below:

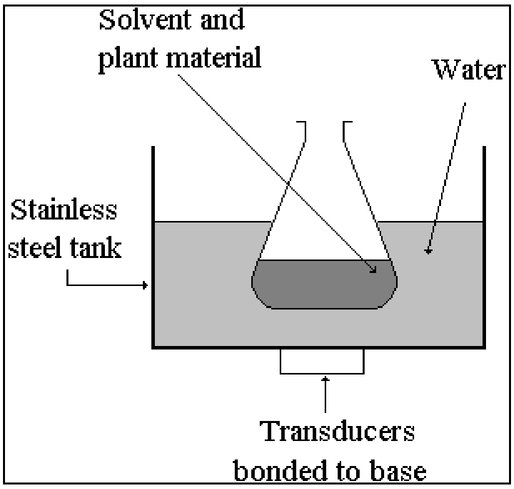

Figure 2.1: Experimental setup for indirect extraction using a cleaning bath (Vinatoru, 2001).

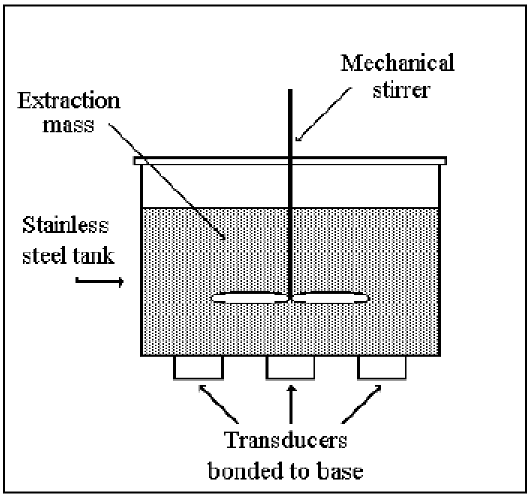

Figure 2.2: Experimental setup for direct extraction using cleaning bath (Vinatoru, 2001).