ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

OPTIMIZATION OF AQUEOUS ENZYMATIC OIL EXTRACTION FROM SAFFLOWER VIA RESPONSE SURFACE METHODOLOGY

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Programme : Chemical Engineering

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SULU FAZ EKSTRAKSİYONU İLE ASPİR TOHUMU YAĞI ÜRETİMİNDE ENZİM ETKİSİ VE OPTİMİZASYONU

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FOREWORD

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ABBREVIATIONS

AE	**:** Aqueous Extraction
AEE	**:** Aqueous Enzymatic Extraction
ANOVA	**:** Analysis Of Variance
AV	**:** Acid Value
2-D	**:** Two Dimensional
3-D	**:** Three Dimensional
FFA	**:** Free Fatty Acid
FAC	**:** Fatty Acid Composition
IV	**:** Iodine Value
PV	**:** Peroxide Value
R^2	**:** Correlation Coefficient
RI	**:** Refractive Index
RPM	**:** Rate Per Minute
RSM	**:** Response Surface Methodology
SV	**:** Saponification Value
UM	**:** Unsaponifiable Matter
TAG	: Triachylglyceride

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OPTIMIZATION OF AQUEOUS ENZYMATIC OIL EXTRACTION FROM SAFFLOWER VIA RESPONSE SURFACE METHODOLOGY

SUMMARY

The aim of this study was to extract good quality oil from safflower seeds by an economical and environmentally-friendly process. For this purpose, aqueous enzymatic oil extraction of safflower assisted by commercial enzymes Alcalase 2.5L and Celluclast 1.5L was investigated and the reaction parameters were optimized via response surface methodology. Although, aqueous enzymatic treatment aided by Alcalase 2.5L resulted in significantly higher oil amounts, Celluclast 1.5L was chosen for the optimization since the effects of the operational variables on the oil amount were more drastic when Celluclast 1.5L was used. Also, the physicochemical properties of the oils extracted by Soxhlet extraction and aqueous enzymatic extraction were analysed and compared in respect to their acid value, saponification value, unsaponified matter, free fatty acid composition and refractory index.

For experimental design, preliminary experiments were established to evaluate the feasibility of enzymatic treatment of safflower and to select the operational range of the most important and effective variables. For this purpose, sub-optimal extraction conditions including enzyme type and amount, (0.5 to 3.0 mL enzyme⁄3 g substrate), buffer solution pH (4 to 8), incubation time (4 to 8 h) and temperatures (30 to 60 °C), churning rate (100 to 400 rpm), seed to buffer solution ratios (1:3 to 1:9 w⁄v), particle size (<0.6 and 0.6-1 mm), cenrifugation conditions and separation methods were varied to determine their influence on the oil amount.

In this research, a three-factor, three-level cubic central composite design requiring a total of 17 design points with 3 centre points (0,0,0) was employed for the optimization of aqueous enzymatic oil extraction. The combined effect of enzyme amount (0.5, 1.5, 2.5), buffer solution pH (4, 5, 6) and incubation temperature (30, 40, 50) (independent variables) on oil amount (response) was evaluated by this method. The obtained experimental data were computed by using Statistica 7.0 software to fit the second-order polynomial model predicted for optimization.

As a result of the optimization, the maximum oil amount and yield were 33.3 (% w⁄w) and 79.7 (% w⁄w), respectively. Incubation temperature was the most significant factor on the oil amount extracted followed by enzyme amount and buffer solution pH. The optimum conditions were determined as follows: temperature, 48.3 °C; enzyme amount, 0.74 mL enzyme⁄3 g substrate (0.6321 EGU⁄3 g substrate); pH, 4.84. At this critical point, 28.2 (% w⁄w) oil amount was observed. The predicted critical values were experimentally verified and an oil amount of 27.1±0.9 (% w⁄w) was achieved. The experimental data for oil extraction yield obtained with Celluclast 1.5L correlated very well with process parameters, resulting in a model with high correlation coefficient for the oil extraction amount (R^2 = 0.9866).

Finally, it was observed that the enzyme treatment did not have any determining effect on the physicochemical properties of the resulting oil.

SULU FAZ EKSTRAKSİYONU İLE ASPİR TOHUMU YAĞI ÜRETİMİNDE ENZİM ETKİSİ VE OPTİMİZASYONU

ÖZET

Bu çalışmada aspir tohumundan çevre dostu ve ekonomik bir yöntemle kaliteli yağ üretimi hedeflenmiştir. Bu amaçla, Alcalase 2.5L ve Celluclast 1.5L enzimleri varlığında aspirden sulu fazda enzimatik ekstraksiyon yöntemiyle yağ eldesi incelenmiş ve reaksiyon parametreleri tepki yüzey metodu kullanılarak optimize edilmiştir. Alcalase 2.5L enzimi varlığında yürütülen ektraksiyon işleminde daha fazla miktarda yağ elde edilmesine karşın Celluclast 1.5L enzimi kullanıldığında reaksiyon parametrelerinin yağ miktarı üzerinde daha etkili olması sebebiyle Celluclast 1.5L ile optimizasyon gerçekleştirilmiştir. Soxhlet ve enzimatik ekstraksiyonla elde edilen yağların asit değeri, sabunlaşma değeri, sabunlaşmayan madde miktarı, serbest yağ asidi bileşimi ve kırılma indisi gibi fizikokimyasal özellikleri analiz edilerek kıyaslanmıştır.

Deneysel tasarım için, aspir tohumu enzimatik yağ ekstraksiyonun yapılabilirliğini incelemek ve en etkili reaksiyon parametrelerini belirleyebilmek için ön deneyler yapılmıştır. Bu amaçla, enzim türü ve miktarı (0.5-3 mL enzim/3 g substrat), tampon çözelti pH'ı (4-8), inkübasyon süresi (4-8 sa), sıcaklık (30-60 °C), çalkalama hızı (100-400 dev/dak), tohum miktarı/tampon çözelti oranı (1:3-1:9 ağ./hac.), tanecik boyutu (<0.6 mm ve 0.6-1 mm), santrifüj koşulları ve ayırma yöntemleri gibi ekstraksiyon parametreleri değiştirilerek yağ miktarına etkileri incelenmiştir.

Bu çalışmada, sulu ortamda enzimatik ekstraksiyon optimizasyonu için 3 merkezli 17 tasarım noktasından oluşmuş 3 faktör ve 2 seviyeli kübik merkezi kompozit tasarım uygulanmıştır. Enzim miktarı (0.5, 1.5, 2.5 mL enzim/3 g substrat), sıcaklık (30, 40, 50 °C) ve tampon çözelti pH'ının(4, 5, 6) (bağımsız değişkenler) yağ miktarı (bağımlı değişken, tepki) üzerindeki etkileri bu metodla incelenmiştir. Reaksiyon parametreleri ile tepki arasındaki ilişkiyi veren ikinci dereceden polinomal bir denklem oluşturulmuş ve bu öngörülen denklemin elde edilen deneysel verilerle uygunluğunu saptamak için Statistica 7.0 programı kullanılmıştır.

Yapılan deneyler sonucu en çok % 33.3 oranında (% 79.7 verim) yağ elde edilmiştir. Yağ verimi üzerinde en etkili faktörlerin sırasıyla inkübasyon sıcaklığı, enzim miktarı ve tampon çözelti pH'ı olduğu saptanmıştır. Tepki yüzey metodu ile bağımsız değişkenler için belirlenen optimum noktalar 48.3 °C, 0.74 mL enzim/3 g substrat ve 4.84 tampon çözelti pH'ıdır. Optimum noktada yürütülen deneyler sonucunda teorik olarak beklenen % 28.2 lik yağ miktarına karşılık % 27.1±0.9 lik yağ miktarı bulunmuştur. Yağ miktarı için elde edilen deneysel veriler ile model denklemden elde edilen tahmini tepki değerleri arasındaki lineer bağlantının korelasyon katsayısı 0.9866 olup model denklemin güvenilirliğini desteklemiştir.

Sonuç olarak, sulu ortamda enzimatik extrasiyon yöntemi ile elde edilen bu yağın fizikokimyasal özelliklerinin solvent ekstraksiyonu ile elde edilen yağla benzer özellikler gösterdiği belirlenmiştir.

1. INTRODUCTION

Oilseeds are those crops in which energy is stored mainly in the form of oil. Some oil crops such as peanut or sunflower can be used directly as a food, but others are exclusively processed to obtain oil and meal. The production of oil crops has expanded rapidly in response to the growing world population and rising living standards. In addition, technological advances have led to higher production levels and improvements in product quality and versatility. Depending on the use of oil, oilseeds can be classified into seeds which contain edible vegetable oil and those which contain non-edible oils. Safflower oil can be used for both purposes. Safflower is increasing in value day-by-day and in the past it was used primarily as a source of dye, a food coloring, a cosmetic, or for medicinal purposes. In recent years, it is widely utilized in food products such as in salad oil, hydrogenated fat, margarine, mayonnaise and in several types of processed food where a high polyunsaturated fatty acid content is desired and the protein-rich meal is used as a source of protein for animal feed and humans. In addition, it is used in industrial products such as alkyd resins, biodiesel, paints and varnishes as a drying agent.

Vegetable oil is traditionally produced by screw pressing followed by extraction with an organic solvent; or alternatively by using solvent extraction alone. The most common solvent used is *n*-hexane. Although *n*-hexane gives a high yield, the process has the intrinsic disadvantages of poor quality of protein in meal, high investment, and energy requirements. Moreover, *n*-hexane can be emitted into the atmosphere during the extraction and recovery steps, where it can react with other pollutants to produce ozone and photochemical oxidants, which can adversely affect the environment. To solve the problems associated with the use of *n*-hexane, aqueous and enzyme-aided extraction processes that may also result in edible protein and good quality oil are being investigated as alternative routes. The aqueous extraction process has been traditionally used in many developing countries, with the water flotation method. To improve oil extraction yields, to reduce the by-products, and to

undertake extraction under milder processing conditions, some enzymes, especially carbohydrases and proteolytic enzymes, have been added to the extraction medium. Enzyme-assisted aqueous processes are potentially useful to the edible oil industries due to their high specificity and low operating temperatures. In comparison with solvent extraction, the enzyme-aided process operates under milder conditions, such as lower temperature resulting in superior quality oil and protein rich meal. However, this process has certain limitations, the major ones being lower efficiency of oil extraction, provision for de-emulsification, enzyme costs, and the treatment of aqueous effluents.

Enzyme-aided aqueous extraction has been practically applied to extract oil from several oilseeds and fruits. This technology has successfully been developed on a pilot scale for extracting coconut oil, rapeseed oil, and olive oil. Other oil-bearing materials treated with enzymes for oil extraction include melon seeds, canola, cocoa fat, *Jatropha curcas* seeds, apricot seeds, cottonseed, wheat, soybean, ricebran, sunflower kernels, and peanut.

As a result, enzyme-aided aqueous extraction processes can be advantageous for extracting oil and other components from oil-bearing material, especially when environmental and safety issues are considered. Several researchers have already reported the application to the aqueous process of food-grade enzymes, for extracting oil from various oilseeds or fruits. In principle, it is possible to improve the extraction yield by using enzyme-aided aqueous extraction process. The oil quality obtained is also reported to be superior, particularly with respect to color and free fatty acid content. However, pilot scale investigation should be undertaken to evaluate the commercial potential of the process with respect to the extraction and separation steps. Furthermore, investigations are needed to simplify the oil recovery steps and make the process commercially more attractive.

2. LITERATURE REVIEW

Safflower, *Carthamus tinctorius* L., which is also known as bastard saffron, false saffron, kardi, kusumb and safflor has a long history of cultivation. The species is believed to be indigenous to Southern Asia but has long been cultivated in China, the Near East, Northern Africa and Europe. For much of its history and until recently, safflower was used primarily as a source of dye, a food coloring, a cosmetic, or for medicinal purposes. Dried safflower florets (from which carthamine, a dye was extracted) were commonly used as an adulterant or substitute for colorful saffron. Later the introduction of other more stable dyes replaced this use for the safflower plant. Safflower was a relatively insignificant local oilseed crop until the early 1950s, when higher yielding oil-bearing varieties were developed and it was established as a source of drying agent for surface coatings, paint and varnish. While it had become known as an edible oil during pre-Christian times in Mesopotamia, it was only in more recent times that it began to be used in India as an edible oil, and it was not until the middle of this century that it began to enter world commerce, first as an industrial oil and then as an edible product. Although it constitutes only about 0.5% of the total oilseeds production of the world, safflower oil is considered as a premium cooking oil owing to its high content of linoleic acid, high iodine value, light yellow color and characteristic pleasant flavor [1,2,3].

Safflower is one of humanity's oldest crops, but generally it has been grown on small plots for the grower's personel use, and it remains a minor crop with world seed production**.** Over 60 countries grow safflower, but over half is produced in India. The important safflower growing countries, besides India, are the USA, Mexico, Ethopia, Spain, the Turkic Republics and Australia. China has a significant area planted to safflower, but the florets are harvested for use in traditional medicines and the crop is not reported internationally [4]. World safflower production by country in 2006 is shown in Table 2.1.

Table 2.1: World safflower production by country in 2006 [5]

Countries	Harvested area (ha)	Production (tonnes)	Yield (kg/ha)
India	350,000	129,000	369
Mexico	85,000	212,765	2,503
USA	64,350	79,730	1,239
Ethiopia	72,000	38,000	528
Kazakhstan	63,000	76,105	1,208
Australia	30,000	50,000	1,667
Argentina	30,000	18,000	600
Kirgyzstan	25,293	22,510	890
Uzbekistan	22,720	8,260	364
Tanzania	15,000	5,000	533
China	15,000	5,000	333
Turkey	165	150	909
World	774,718	675,831	872

It is believed that cultivated safflower was first introduced into Turkey in the 1930s by Turkish immigrants. Attempts to commercially produce safflower were slowly introduced in the 1950s. From 1972 to 1977 attempts were made to adapt safflower on commercial scale, but the varieties available were too low in oil. Currently, Turkey has a major deficit in oilseed production and only 40 % of oil seed needs are met by crops grown in Turkey. Today, Turkey imports about 50% of vegetable oil consumed. In order to reduce deficiency in oil production, oilseed crop production areas and oil yield should be increased or alternative oil crops should be introduced. Safflower (Turkish: Aspir) which has a potential to meet much of Turkey's oil demand is one of the alternative oil crops. In addition, new energy sources like biofuels are being researched because Turkey's own energy supply is insufficient. Although the best oilseeds for biodiesel production in Turkey are safflower and rapeseed, 70 % of biodiesel is produced from imported palm oil or its derivatives. Regarding growth conditions, safflower is not selective and is more tolerant to

draughts, low temperatures, and salinity stress than other oil crops. Since it is resistant to draughts, it could be grown successfully on the dry lands of Central Anatolia and surrounding regions which have insufficient precipitation such as Ankara, Eskisehir, Konya and Cankiri provinces. Safflower is even less known than canola farming but safflower production is more beneficial. Its cultivation is much easier and its cost of production is lower than wheat, sunflower, and rapeseed. However, Turkey is a small scale safflower producer. In recent years, due to its small acreage, lower economic importance, low yield and lack of research about adaptability of new cultivars, improvement and cultivation techniques, safflower production has been limited. Total safflower production was 1600 tonnes in 1976 which decreased to 20 tonnes in 2000. Acreage has varied from nearly 1,000 to 7,000 ha. Acreage and yield trends of safflower over the last 50 years are depicted in Table 2.2 [4,6].

Table 2.2: Safflower production by year in Turkey [4]

Years	Harvested area (ha)	Production (tonnes)	Yield (kg/ha)
1950	1,073	765	712
1970	1,170	900	769
1990	146	124	849
2000	30	18	600
2004	165	150	909
2007	4,000	4,000	1,000
2008	7,000	-	-

Safflower is a plant of desert origins with waxy leaves and a relatively thick hull. It is an annual oilseed crop which has spiny and spineless varieties. Generally, varieties with reduced or absent spines have been lower in oil content than spiny types. The common white, normal-hull high-linoleic types usually contain 20% to 45% oil. Floret color varies from whitish yellow to red-orange (carthamin), the most common being deep yellow (carthamidin). A water-soluble carthamin and carthamidin are extracted from florets to obtain dye.

A safflower seed consists of a tough fibrous hull that protects a kernel with two cotyledons and an embryo. The kernel constitutes about 51 to 62% while the hulls make up about 38 to 49% of the total seed weight. The approximate compositions of kernel and hull indicate that the oil, protein, and ash of safflower seeds are concentrated in the kernel while the fiber is found in abundance in the hull portion. These results show that more than 96% of the lipids and proteins of safflower seed are located in the kernel. The proportion of hull is predominant and has been a disadvantage to commercial production as it dilutes both oil and protein contents of the safflower seed. Also, a higher proportion of hull in press meal prevents its use as animal feed and increases the cost of oil extraction. Safflower seeds are normally cream to white, but breeding has resulted in a greater variation in color, ranging from normal hull to thin hull. Research has been aimed at creating a thinner hull to increase oil content. [1,2]

On the basis of the principal fatty acid present in safflower, the varieties are often grouped as high-linoleic, high-oleic and high-stearic acid types. Linoleic level decreases proportionally as oleic level increases. Although plant types differing in the hull content and the major fatty acid content are available in safflower, the most common wild safflower cultivated on a commercial scale is a white, normal-hull, high-linoleic acid type that contains more than 70% linoleic acid in its oil. However, recently some cultivated safflower varieties have been developed with characteristic fatty acid compositions such as high-oleic types, with more than 70% oleic acid in their oil. That is to say an oil in which the normal levels of linoleic and oleic fatty acids are reversed is produced. As a result, the oil has an improved oxidative stability in the cooking process over normal safflower oil due to the replacement of polyunsaturates with monounsaturates. Fatty acid composition of the oil for both high-oleic and high-linoleic types of safflower have been found to be very similar. High-oleic safflower oil displays most of the same characteristics as the high-linoleic type, except for its fatty acid structure. The high-stearic acid types contain about 5 to 10% stearic acid in their oil [1,2,3].

2.1 Safflower Oil

Safflower oil has a pale or golden yellow color and is bland or has a slightly nutty flavor depending on the method of processing used. It is a drying oil, intermediate

between soybean and linseed in total unsaturation. The important physicochemical properties of safflower oil are summarized in Table 2.3 and Table 2.4. Safflower oil exhibits a higher refractive index, specific gravity, density, iodine value and linoleic acid content than most of the other common edible oils [1].

Table 2.3: Physical properties of safflower oil

Color (Gardner)	Specific gravity (25 ºC)	Refractive index (25 ºC)	Density (25 ºC)	Flash point (ºC)	Melting point (ºC)	Solidification Point (ºC)
8-10	0.92	1.473-1.476	0.92-0.94	148.8	-5	-13 to -20

Regarding chemical composition, the factors that affect the concentration of various chemical constituents in seeds include genetic backround of the variety, geographic region, and agronomical practices.

Table 2.4: Chemical properties of safflower oil [2,7]

SV (mg KOH/g oil)	IV (Wijs)	PV (mEq/kg)	AV (mg KOH/g oil)	UM (% w/w)	FFA (% as oleic)	FAC	
						S (%)	US (%)
186-194	130-150	0-1	0.4-10	0.3-1.3	0.15-1.09	5-10	90-95

SV, saponification value; IV, iodine value; PV, peroxide value; AV, acid value; UM, unsaponifiable matter; FFA, free fatty acid; FAC, fatty acid composition; S, saturated; US, unsaturated

The oil consists of mainly TAGs or neutral lipids. Other constituents like phosphatides (0.5%), and unsaponifiable lipids (0.3-1.3%) are present in minor quantities. The total content and type of phosphatides do not differ in normal and high-oleic seed types. The phosphatides have been implicated in the problem of dark color formulation occurring in extracted and heated crude safflower oil. The unsaponifiable fraction in safflower oil mostly constitutes sterols and terpens which are higher in germ and hull than cotyledons. The crude safflower oil contains 0.63% total sterols while the refined oil contains about 0.56%.

The ranges for various fatty acids in common high-linoleic acid types are demonstrated in Table 2.5. The oil of commercial safflower cultivars contains 55 to

81% linoleic acid and 7 to 42% oleic acid as major fatty acids, followed by stearic (1-10%) and palmitic acid (2-10%) as minor fatty acids. Other fatty acids such as $C_{14:0}$, $C_{14:1}$, $C_{15:0}$, $C_{16:1}$, $C_{17:1}$, $C_{18:3}$, $C_{20:0}$, $C_{20:2}$, $C_{21:0}$, $C_{22:0}$, $C_{24:0}$, $C_{24:1}$ are present in safflower oil in very minor traceable amounts. The unsaturated fatty acids make up about 90% of the total safflower oil [1]. The composition of safflower oil is largely made up of linoleic fatty acid with a very low level of linolenic acid, which results in an ideal drying oil in paints and varnishes because of its non-yellowing characteristics. Besides linoleic acid being an essential fatty acid, the nutritional quality of safflower oil is considered to be higher compared to most other edible oils. Linoleic, principle fatty acid of safflower, is an essential fatty acid that cannot be synthesized in the human body. Safflower oil is the natural raw material for the production of conjugated linoleic fatty acid. Hence, it has been used in food products where a high polyunsaturated fatty acid content is desired. The oil is widely utilized in salad oil, hydrogenated fat, margarine, mayonnaise and in several types of processed food. Fresh safflower salad-grade oil has excellent flavor and odor characteristics, and because it lacks linolenic fatty acid, it does not display fishy or beany odors. On the other hand, flavor stability has been a constant problem with products containing appreciable quantities of safflower oil because of high-linoleic fatty acid content. Therefore, market demand has drastically shifted from the traditional high linoleic oils to high-oleic oil in recent years [1,2,3].

Table 2.5: Typical fatty acid composition of linoleic and oleic safflower

Safflower variety	Palmitic acid (16:0)	Stearic acid (18:0)	Oleic acid (18:1)	Linoleic acid (18:2)	Other
Linoleic type	5.2	1.5	15.0	77.0	1.3
Oleic Type	4.5	1.5	77.0	15.0	2.0

The total protein content in seeds is between 11 to 24% and most of the storage proteins are concentrated in the kernel. Since the safflower seeds are extracted for oil, the meal obtained as a by-product in the oil industry carry most of the seed proteins. The expeller press meal contained 55.5% protein while the commercial desolventized meal contained 45%. Also, the heat generated during expeller pressing

(90 ºC) does not influence the protein solubility while the heat employed (110 ºC) in the desolventizer significantly impairs the protein solubility in safflower. This can be attributed to heat denaturation of storage proteins. Safflower proteins are rich in aminoacids including glutamic acid, aspartic acid and their amines. On the other hand, the proteins are deficient in lysine, methionine, threonine and isoleucine. The protein isolates prepared from different meals and by different methods exhibit similar aminoacid composition. The whole safflower seed contains 18.5% carbohydrates and 21.7% crude fiber. The hulls are a major fiber and carbohydrate source in safflower.

Regarding vitamin E or tocopherol content, safflower oil contains low amount of γ-tocopherol, indicating its poor antioxidant ability. Among the various common edible oils, it contains more α-tocopherol than corn, peanut, rapeseed, soybean or sesame oils. Since safflower oil contains a higher proportion of linoleic acid than most of the common edible oils, its α-tocopherol content is important in terms of α-tocopherol:linoleic acid ratio. α-tocopherol:linoleic acid ratio is low, 0.28 as against 0.84 for sunflower oil and 1.0 for cottonseed oil [1]. This indicates a need to lower the level of linoleic acid in order to increase its shelf life as the oxidative stability of crude safflower oil precludes storage for indefinite periods before processing. Thus, it is obvious that safflower oil with its high linoleic acid content and a low level of antioxidants is not particularly stable [3]. The equal oleic:linoleic and high-oleic type cultivars may exhibit higher α-tocopherol:linoleic acid ratios. The total ash content in safflower seed ranges from 2.0 to 3.5%. Minerals determined in the meal were P, Mg, Ca, Fe, Zn and Mn. The safflower meal can be a rich source of dietary minerals, particularly Ca, P and Fe. As for the antinutritional factors, the use of safflower meal in human consumption is limited, primarily due to the presence of fibrous hull and two phenolic glucosides which exhibit cathartic activity and a bitter taste. Methanol extraction removes these glucosides, residual fat and some carbohydrates which increase the protein content in the debittered meal [1].

2.2 Oil Recovery from Safflower Seeds

Much of the safflower processed in India in the past was crushed by a mortar-and-pestle like devices called *ghani* which is an example of warm mechanical pressing [2]. Today, the methods of recovering oil from safflower follow very simple principles: mechanical press (expeller) extraction, prepress solvent extraction and solvent extraction. The preferred extraction process depends upon the quantity of meal that can remain in the meal, how much meal protein denaturation is allowed, the investment capital available, and how restrictive the environmental laws are regarding emissions of organic compounds. The prime objective in using any of these methods is to recover the maximum amount of an unchanged oil that is as free of impurities as possible and to produce co-products of maximum value.

As for the preparation for pressing or extraction, the safflower seeds must be cleaned to remove any impurities as foreign matter reduces oil and protein yields, adversely affects oil quality, and increases damage to the processing equipment. High-capacity dry screeners are used to remove all impurities by utilizing a combination of screens and aspiration. Following cleaning, the seeds are dehulled or decorticated. The hulls which have a low oil content will absorb and keep oil in the press meal, and reduce the capacity of the extraction. However, safflower seeds sometimes are not dehulled because their small size makes it difficult to remove the hulls efficiently. The oil extraction process is facilitated by the reduction of the seed to small particles by grinding or flaking which causes sufficient oil-bearing cell rupture to liberate the oil for ease of extraction. The next step is heating or cooking which results in the complete breakdown of the oil-bearing cells, coagulation of the proteins to facilitate the oil and meal separation, insolubilization of phospholipids, increased fluidity of the oil at higher temperatures, inactivation of enzymes, destruction of molds and bacteria, and drying to a desired moisture content.

Before the expander was introduced, cooking was the final oilseed conditioning step. The expander is a low-shear extruder that heats, homogenizes, and shapes oilseeds into porous pellets with a high bulk density. In some cases, expanders are used instead of prepress solvent extraction as they enable the production of intact pellets for direct solvent extraction [3].

Regarding the products obtained from each process, mechanical press extraction is simpler and safer but less efficient than solvent extraction. That is to say more oil is recovered from the safflower seed by solvent extraction. It leaves 3-6% oil, whereas solvent-extracted meal contains 0.5-1.5% oil [8]. On the other hand, important nutrients are missing in the meal from the solvent extraction; therefore, meal from mechanical pressing is undoubtedly more valuable as animal feed or a source of isolated protein for human dietary than those from extraction. In addition, the final portions of the solvent agent can only be removed in a costly fashion from the meal after extraction. The solvent extraction agent not only extracts oil from safflower seed, but also resins, dyes and bitter elements that influence the quality of the oil. For this reason, solvent extraction hardly comes into question for edible oils.

After pressing as well as extracting, the oil obtained still contains water, mucilage materials and other impurities that must be eliminated by refining, bleaching and deodorizing processes which include decanting, filtering, and centrifuging [7].

2.2.1 Mechanical press (expeller) extraction

Expeller pressing mechanically squeezes the oil from the seed. In the screw press, the cooked flakes are separated into crude oil and press cake. The press cake is then ground into meal and sold as a protein source. After settling and filtration to remove fine particles, the crude oil is then transferred to oil refiners for further processing into finished product formulations [3].

The safflower seed is generally first pressed without heat. This first cold pressing yields the most valuable edible oil called “virgin oil”. After the cold pressing, the seed is warmed up and pressed for the second time. While up to 20% of the oil content remains behind with the unwarmed seed, warm seed can be degreased up to 5-10%. With warm pressing, however, substances get into the oil that can unfavorably influence its color, smell, and taste. However the advantage of the warm pressing is that the oil becomes more fluid, that the proteins coagulate and mucilage materials and plant gums fall out for the most part. Warm pressing is preferred when higher oil yields are aimed while cold pressing is used if a particular oil quality is required [7].

There are two types of expellers: screw press and hydraulic press. A mechanical screw press extracts oil by applying extremely high pressure to seed material by

means of a decreasing volume [3]. In the past, hydraulic presses which did not require heat or chemicals were used, but they were replaced with continuous screw presses later on for the mechanical extraction of safflower seeds in various parts of the world [2].

2.2.2 Prepress solvent extraction

In many cases, mechanical press extraction and solvent extraction processes are combined and called “prepress solvent extraction”. Prepress solvent extraction removes a portion of the oil with expellers, and the remainder of the oil is extracted with an organic solvent, usually *n*-hexane. During pre-pressing, the expeller is choked so that less pressure develops and less oil is recovered. The safflower seeds are first squeezed out partially, and this way a high-grade edible oil is obtained. Subsequently, the remaining oil in the meal which is used for technical purposes, is solvent-extracted using the same procedure as for direct solvent extraction, but then no protein rich meals are obtained. The pre-pressed and solvent-extracted oils are usually mixed before refining. The advantages of this system are that the capacity of the screw press is increased and a smaller solvent extraction plant is required to recover the oil from the meal [3,7].

2.2.3 Solvent extraction

Since the mechanical press extraction led to low oil recovery, the desire to produce a higher oil recovery was deemed necessary. Therefore, solvent extraction of oil in this manner came into question . Direct solvent extraction removes the oil directly from conditioned safflower seeds with an organic solvent. In the 1940s, high oil content of safflower seed flakes caused them to disintegrate into fines during the extraction process. This problem was solved by using a low-shear extrusion method, with equipment identified as expanders [3].

The extraction process is based on a counter-current flow of the solvent and the seed in the extractor: solids running in one direction while being washed by solvent passing in the other. The extraction rates are higher than other extraction methods. The common solvent used is *n*-hexane of 65 ºC boiling point [1]. Even though elevated temperatures reduce oil viscosity and enhance diffusion, the hexane vapor pressure limits the practical operating temperatures of the extractor and its contents to approximately 50-55 ºC. Separation of the oil and solvent is accomplished by

conventional distillation methods. The full miscella, which is the solvent and oil mixture, is distilled to free the oil from the solvent. The recovered solvent is separated from the accumulated moisture and reused in the solvent extraction operation. The hexane-free oil is cooled and filtered before storage or further processing [3].

2.2.4 Aqueous extraction

Industrial processes for the extraction of edible oil from oilseeds generally involves a solvent extraction step, sometimes preceded by mechanical pressing. Mechanical pressing is a less efficient process which lead into low oil recovery (40–60%). Solvent extraction, although its recovery is in the 90–98% range, has the intrinsic disadvantages of poor quality of protein in meal, high investment, and energy requirements. The commercial hexane used as the most common solvent for oil extraction is listed among hazardous air pollutants associated with neurological and respiratory disorders caused by prolonged exposure. Hence, there is a need to explore alternative safe and efficient oil extraction processes that may also result in edible protein and good quality oil. In the past, safety considerations on the use of organic solvents accelerated investigation of aqueous extraction (AE) and aqueous enzymatic extraction (AEE) but these were unsuccessful due to the low oil yields. Recently, interest in aqueous extraction has been revived because of the need for environmentally cleaner alternatives for edible oil extraction [9,10].

The AE process, in which oil extraction is based on the insolubility of oil in water rather than on the dissolution of oil, is one such alternative. In AE, oil and protein are extracted from the high-fibre solids and the extraction mixture is centrifuged to produce oil-rich (free oil and cream) emulsion, oil-protein lean spent solids and a protein-sugar rich aqueous phase (skim). AE offers several advantages over conventional solvent extraction: less capital investment, inherently safe operation, and simultaneous production of edible oil and protein rich fraction with less protein damage. The challenges when using this process are to improve the efficiency of oil extraction, to effectively de-emulsify the difficult-to-break cream in order to recover free oil when emulsions are formed, and to develop high value uses for the dilute protein rich aqueous effluent (skim).

The AE process has been traditionally used in many developing countries with the water flotation method used for extracting coconut and palm oil. This method involves heating seeds, grinding with or without water, and boiling with water to liberate the oil, which appears on the surface. The oil is collected and heated to remove moisture. Oil extraction yields around 50% are generally considered satisfactory for traditional non-commercial processes.

AE has been investigated in the laboratory to increase extraction yields, especially for coconut and peanut, which have relatively high oil content. AE has also been applied to other sources of oil such as lupin seed, palm kernel, sunflower kernel, soybean, and rice bran. This process can be used to extract not only oil but also high quality proteins. To improve oil yields and to undertake extraction under milder processing conditions, some enzymes, especially carbohydrases and proteolytic enzymes, have been added to the extraction medium [9].

2.2.5 Aqueous enzymatic extraction

Aqueous enzymatic extraction (AEE) is another alternative eco-friendly process based on simultaneous isolation of oil and protein from oilseed by dispersing ground seed in water and separating the dispersion by centrifugation into oil, cream, aqueous and solid phases [11].

The utilization of enzymes in food processing has long been recognized with a view toward achieving high product yields, reducing by-products, and avoiding severe operational conditions. The use of cell wall–degrading enzymes in aqueous extraction to treat oil-bearing seeds and fruits has also been considered in recent years. AEE processes are potentially useful to the edible oil industries due to their high specificity and low operating temperatures. Enzyme applications in edible oil include facilitating pressing, increasing the oil yield of solvent extraction, and facilitating the aqueous extraction. It is suggested that enzymatic treatment of seeds prior to oil extraction could improve the extraction rate and oil quality [9,11].

The cell wall of plants consists mainly of pectic substances, cellulose, hemicellulose and lignin [12]. The basic principle is to digest the cell walls of oil-bearing seeds with suitable enzymes to extract oil, protein, and other components present in intracellular vacuoles, under milder processing conditions than AE, ie. at lower temperatures or pH values around 8. The presence of certain enzymes during

extraction enhances oil recovery by breaking cell walls (cellulases, hemicellulases and pectinases), oil bodies (proteases) and a partial decomposition of matter fibres, therefore favoring the oil flow and increasing the extraction yield. Proteolytic enzymes seem to be effective in hydrolysing the oleosins, the lipophilic protein surrounding lipid bodies, thereby decreasing the surface activity of oleosin and enabling removal of lipid. This technology has successfully been developed on a pilot scale for extracting coconut oil, rapeseed oil, and olive oil. Other oil-bearing materials treated with enzymes for oil extraction include melon seeds, canola, cocoa fat, *Jatropha curcas* seeds, apricot seeds, cottonseed, wheat, soybean, ricebran, sunflower kernels, and peanut [9,11,13].

The enzyme selected and its activity must be appropriate to the oilseed or fruit to act with a high degree of specificity. In the case of oilseeds with high cellulose and hemicellulose content, cellulase and hemicellulase are necessary. Some of the enzymes investigated include α-amylase, pectinase, cellulase, hemicellulase, and protease. A single enzyme or a mixture of enzymes can be used. The extraction yields using enzyme mixtures are usually found to be higher, especially in the cases of coconut, Shea tree kernel and avocado. When the cost or availability of commercial enzymes is a major concern, crude enzyme preparations containing several enzymes from selected microorganisms can be used. In such cases, it is necessary to ensure that the preparation is free of lipase or any related enzyme.

Enzyme-treated oilseeds have been observed by microscopic methods. It has been reported that soybean treated with cellulase (Celluclast) and an enzyme mixture consisting of cellulase and hemicellulase (Multifect) had smaller particle size than untreated samples. Enzymes were also found to degrade cell walls, which caused the dispersion of the cytoplasmic constituents. On the other hand, mechanical processes only rupture and dislocate the cell wall, and completely damage the inner cotyledon, causing release of the cytoplasmic material.

The scheme employed in the AEE process is shown in Figure 2.1. The steps are; pretreatment of oil-bearing material such as drying, grinding, sieving, and boiling, treatment with enzymes, and separation of oil or emulsion and other components.

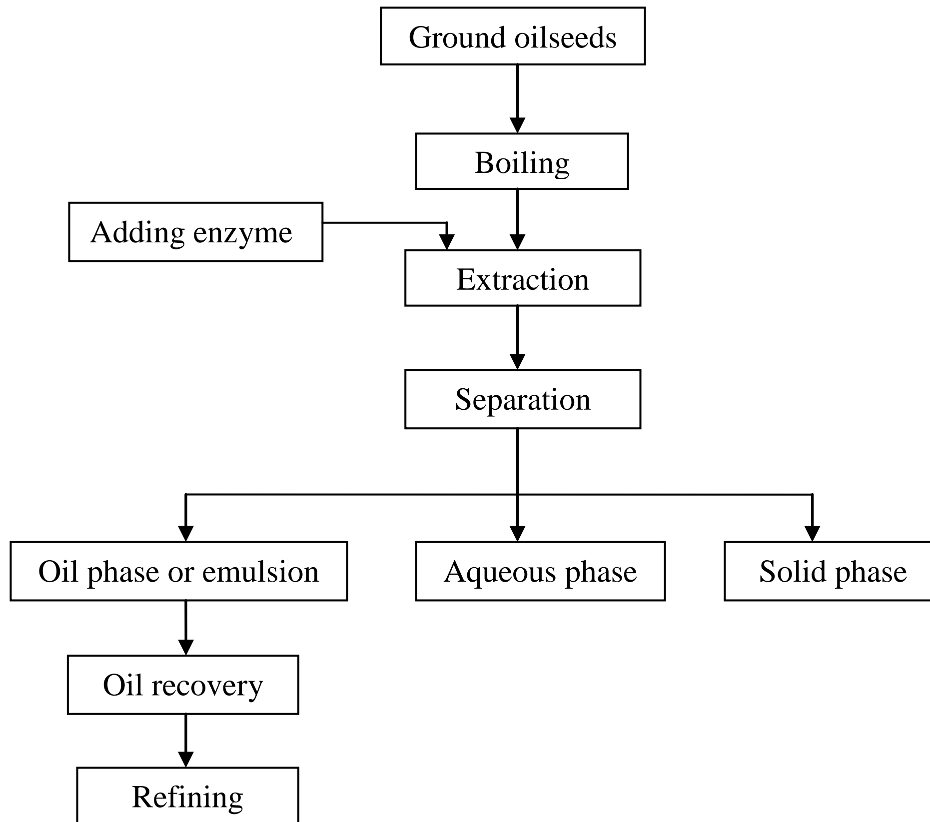

Figure 2.1: Process scheme for AEE.

In comparison with solvent extraction, the enzyme-aided process operates under milder conditions, such as lower temperature. However, this process has certain limitations. The major ones are lower efficiency of oil extraction, provision for de emulsification, enzyme costs, and the treatment of aqueous effluents [9].

2.2.5.1 Factors affecting AEE

In AEE, several factors affect yield and product quality. To enable the action of enzymes, extraction conditions must correspond to those recommended for the use of the enzyme. The key factors affecting extraction, as reported by several investigators, are enzyme composition and concentration, particle size of oilseed/fruit, solid-to-water ratio, incubation temperature, buffer solution pH, churning rate and incubation time [9].

2.2.5.2 Downstream process for AEE

To separate the oil after extraction, most recent studies have involved the use of centrifugation. The mixture is centrifuged at between 1519 and 16,000 *g* at a

temperature of 20 °C, which results in separation into four phases: oil-rich, cream, aqueous and solid phases. Moreover, oil separation can be accomplished using the hot-water flotation method by adding warm water to the extraction mixture. The emulsion layer is allowed to float to the top, collected, and boiled gently to break up the emulsion. Finally, the free oil is decanted. This method is traditionally used in developing countries.

The de-emulsification and product recovery steps essentially determine the viability of aqueous and enzymatic extraction. If emulsions have formed during extraction, they must be broken down to release the oil. Emulsions can be broken down by several methods, as mentioned earlier. The oil in emulsions can also be separated by extraction with *n*-hexane. Freezing and thawing can also be used to break up emulsions. In principle, oil globules in emulsions coalesce to form larger droplets, which are easier to separate by centrifuging [9].

2.2.5.3 Quality of oil recovered by AEE

Several reports suggest that oils recovered by enzyme-assisted aqueous extraction have superior quality. It has been reported that oil obtained by AEE is lighter in color than crude oil obtained by solvent extraction. In the case of coconut oil, the crude oil recovered is clear; therefore, no further refining is required. It has also been reported that corn germ oil obtained by enzymatic extraction had free fatty acid, peroxide, and other oxidation products in the same range of values as oil obtained by *n*-hexane extraction. It was relatively stable due to low content of phosphatides, and it also had lower levels of coloring substances; thus, it required less bleaching earth for purification. Christensen mentioned that the storage stability of olive oil extracted by the enzymatic process was significantly better than that of the conventional press process. The quality of soybean oil from the AEE process in terms of free fatty acid content and peroxide value and its color were also comparable with crude commercial soybean oil and oil from an untreated process [9].

2.2.5.4 Advantages and disadvantages of AEE

There are several advantages and disadvantages of AEE and solvent extraction processes in terms of solvent use, cost, oil yield, by-products and eco-friendliness. Application of aqueous enzymatic process in oil extraction is undoubtedly an

emerging technology in the fats and oil industry since it offers many advantages compared to conventional solvent extraction. Its main advantages are that it is environmentally friendly and does not produce volatile organic compounds as atmospheric pollutants. For instance, it eliminates solvent consumption, which reportedly may also lower investment costs and energy requirements. Also, it enables simultaneous recovery of oil and protein from most oilseeds and process yields oil of good quality. Oilseeds have glucosinates, tannins, sinapin, and the phytic acids that remain in the meal when conventional extraction methods are used. The AEE gives meals that are considerably lower in these undesirable compounds and, therefore, higher levels of meal can be incorporated into feed formulations**.** On the other hand, its major limitations are lower oil yields (in a standard operation it is not uncommon to have approximately 18-25% of the available oil unrecovered in the process), the need for more difficult oil recovery steps, and the production of significant volumes of aqueous effluent. To reduce aqueous effluent and save energy consumption, water used for extraction can be recovered and reused. Furthermore, recycling enzymes with water circulation can cut down on the cost of the enzymes. Of course, it is possible to recycle and reuse the enzyme only if its activity has not dropped significantly during the process. Another disadvantage associated with AAE is the long process time which is necessary for enzymes to liberate oil bodies. Another factor (sometimes neglected) is the use of enzymes which are not commercially available. This prevents the use of the process by other workers [9,14].

Finally, by employing AEE process, the need for further degumming operations is reduced and process allows ready removals of some toxins and anti-nutritional compounds from certain oilseeds. In this sense, some of the needs triggering technology innovation in the oil extraction such as cost savings, environmental and safety concerns, and nutritional issues seem to be achievable by successful development of AEE [14].

The economics of enzyme-assisted aqueous oil extraction has been previously compared with solvent-based extraction, which involves a high capital cost to install. It was determined that if market rates for product oil are high, the enzyme-assisted oil extraction process can compete favorably with the conventional approach; and if immobilized (reusable) forms of enzyme are used, recycling the enzyme can considerably reduce the cost [15].

2.2.6 Literature review of aqueous enzymatic oil extraction

Several researchers have already reported the application to the aqueous process of several enzymes to extract oil from various oilseeds or fruits. In principle, it is possible to improve the extraction yield by using AEE. The low oil recovery in AE and AEE processes has been related to the inadequacies of pre-treatments at disrupting the cellular structure of oilseeds. Mechanical and heat treatments (coagulating the proteins in the lipid bodies and reducing viscosity which allows the oil to flow) have been used to improve the rupture of cell walls, facilitating further enzyme degradation of the cell walls. Much of the research conducted focused on following aspects of the enzyme-aided aqueous extraction process: the influence of extraction parameters on process optimization, downstream processing, and key issues relating to the quality and yield of the oil obtained.

Nyam [10] investigated that the physicochemical properties of oil from Kalahari melon seed following extraction with petroleum ether and aqueous enzymatic methods. Two different enzymes Flavourzyme 1000L and Neutrase 0.8L were separately used. The free fatty acid, peroxide, iodine and the saponification values of the oils extracted using the methods were found to be significantly different. The melting point of the oils extracted was in the range of -18.7 degrees to -17.5 ºC and no significant difference between the oil obtained from solvent and aqueous enzymatic extractions was observed. Enzyme-extracted oil tended to be light coloured and more yellow in colour compared with solvent-extracted oil. The predominant fatty acids in the extracted oils were linoleic acid (62.2-63.1%), with some oleic (16.8-17.1%), palmitic (11.4-12.4%), stearic (7.5-8.1%), linolenic (0.7-1.2%) and eicosenoic (0.3%). Phenolic acids in enzyme-extracted oils were comparable to the solvent-extracted oil. The oils extracted with these two methods were differed in the composition of their phytosterol and tocopherol contents, but no significant difference between the two enzyme-extracted oils was observed.

Ghodsvali *et al.* [12] observed the effects of olive variety (Kroneiki, Iranian Native Oleaginous and Mission), enzyme type (Pectinex Ultra SP-L and Pectinase 1.6021) and concentration (zero, low and high concenteration) on the yield, total polyphenols, turbidity, colour, acidity. peroxide value and iodine value of three enzyme-treated virgin olive oil. The enzyme concentration had a highly significant effect on the yield, colour, turbidity and total polyphenol level of oil, but there were

not significant effects on acidity, peroxide value and iodine value. Colour and phenolic compounds content in the oils showed significant differences between 13.0-62.2% and 13.9-72.6%, respectively, as compared with control. Turbidity was reduced significantly 25.9-67.4%. On the basis of our results, the yield of oil was significantly increased (from 0.9% to 2.4%) by using processing aid. Pectinex Ultra SP-L was more effective than Pectinase 1.06021. In the case of applying Ultra pectinex SP-L, the additional income due to extra recovered oil will be 18.8 times as much production overhead.

Dominguez *et al.* [14] reported that in the case of soybean oil extraction, the oil yields changed drastically when the enzyme-to-seed ratio was varied from 0.1 to 1.0 g enzyme⁄100 g substrate. A moisture content of 50–70% (soybean-to-water ratio about 1:1 to 1:2) in treatment gave maximum yield. Incubation time also had a strong effect on oil yield, with a period of 6 h (hours) reported to be suitable. Smaller particle sizes (<1 mm) also gave higher yields due to improved accessibility of enzyme to the cell wall. When different enzymes were compared, a mixture of cellulase and hemicellulase preparation (Multifect) gave the highest oil yield, 44%. Rosenthal *et al.* [15] improved oil and protein extraction yields from soybean to 58% and 67%, respectively, by using protease (Alcalase). These yields were higher than the values obtained by treatment with cellulase, hemicellulase, and pectinase individually.

Tano-Debrah *et al.* [16] attempted to extract oil from Shea kernel by using protease (Sumizyme AP) and a mixture of cellulase and hemicellulase preparation (Sumizyme C), which resulted in the yield increasing to 72%, compared with only 48% without addition of the enzyme. The optimal conditions were enzyme concentration of 1%, meal dilution of 1:2, and incubation at 30 °C for 4 h. The dilution ratio, i.e., the ratio of material to water, is also important to the enzymatic process because it influences the action of enzymes and the diffusion of the enzymes and hydrolysis products.

Hanmoungjai *et al.* [17] reported that the free fatty acid value of crude rice bran oil obtained from the enzyme-aided aqueous process was significantly lower than for solvent-extracted crude oil. Therefore, lower amounts of neutralizing agent are necessary in the refining steps. Furthermore, the composition of essential fatty acids in enzyme-extracted rice bran oil is comparable with commercial and solvent-extracted oil.

Hanmoungjai *et al.* [17,18] investigated the extraction of oil and protein from rice bran using commercial enzymes. It was found that the oil yield obtained by using commercial protease (Alcalase) was higher than those obtained by using other enzymes. The optimal conditions were incubation with 1% Alcalase at pH 9.0 and a temperature of 50 °C for 1 h.

Jiang *et al.* [19] investigated extraction of oil and protein hydrolysates from peanut by using Alcalase 2.4L and established parameters for hydrolysis, the single-factor and orthogonal test. The optimal processing conditions were as follows: hydrolysis temperature 60 °C, pH 9.5, ratio of material to water 1:5 (w/w), alkaline extraction time 90 min, enzyme amount 1.5% (w/w) and hydrolysis time 5 h. Under these conditions, the free oil and protein hydrolysates yields were 79.32% and 71.38%, respectively. It was found out that total free oil and protein hydrolysates yields were more than 91.98% and 88.21%, respectively by using As1398 enzyme.

Caetano *et al.* [20] mentioned that the combined use of thermoplastic extrusion and enzyme technology in aqueous extraction of sunflower oil improved oil yield. Extrusion and enzyme incubation parameters were established by response surface methodology by using commercial enzymes (Viscoenzyme and Alcalase). Conditions selected for the process were: 70 ºC, 4 h, screw speed 180 rpm at the extrusion stage, dilution ratio of 1:5 and enzyme dosage 0.3% (v/w). Extrusion increased the process yield by approximately 54%, and the laboratory enzyme was more effective than the commercial enzymes for aqueous extraction of the oil. Maximum oil yield from aqueous enzyme extraction was approximately 82 and 70% for the E122-V2000 and commerical cultivars, respectively.

Latif *et al.* [21] investigated the effect of various enzymes on aqueous extraction of canola seed oil and protein. Enzymes tested for their effectiveness in releasing oil and protein during aqueous extraction were Protex 7L, Multifect Pectinase FE, Multifect CX 13L, and Natuzyme. The enzyme-extracted oil content of canola seeds (22.2-26.0%) were significantly higher than that of the control (16.48%). An appreciable amount of protein (3.5-5.9%) originally present in the seed was extracted into the aqueous and creamy phases during aqueous extraction of oil. The physicochemical properties of oils extracted from canola seed by conventional solvent extraction, and aqueous extraction, with or without enzyme addition, were compared. Significant differences were observed in free fatty acid content, specific

extinction, peroxide value, color and concentration of tocopherols (alpha, gamma, and delta). However, no significant variation was observed in iodine value, refractive index (40 ºC), density (24 ºC), saponification value, unsaponifiable matter, and fatty acid composition. The oil quality was better in enzyme extracted oil than in solvent-extracted oil. While the enzymes enhanced the oil extraction, the oil yield was still significantly lower than that obtained by solvent extraction.

Sharma *et al.* [22] evaluated the benefit of using ultrasonic pre-irradiation before extracting oil from almond and apricot seeds by AEE process. The use of a commercial preparation which is a mixture of three proteases gave 75% w/w oil yield from almonds at pH 4.0 in 18 h at 40 °C. The ultrasonic pre-irradiation at 70 W for 2 min increased the yield to 95%, w/w and reduced the extraction time to 6 h. The effect of ultrasonic pre-irradiation on meal morphology could be visually seen by scanning electron micrographs. It indicates development of microfractures and disruption of cell walls in almond powder. With apricot, ultrasonic pre-irradiation also marginally increased the oil yield obtained by AEE to 77% w/w and reduced the extraction time to 6 h. Thus, ultrasonic pre-irradiation step may reduce time required to extract oil from edible oils from plant sources and hence can improve commercial oil production process.

2.3 Regression Analysis

Regression analysis can be used to statistically estimate the relationship between two independent variables and the value of a dependent variable (response). But a simple regression analysis is of poor meaning. It is much more valuable when it is used to check if the outputs of a system are really like the hypothetically predicted ones. It does not prove that the dependent variable is determined by the independent variables, it only states that there is a highly numerically relationship between them.

It is also important to plot predicted values computed according to the model by numerical analysis vs. corresponding observed experimental values. Because it can easily cause errors or lead to inaccurate results when computed results blindly used without checking the plot between independent and dependent variables. For this purpose, R^2 (correlation coefficient) represents whether the values of the response predicted from the empirical model are in good agreement with the observed values in the range of operating variables [23].

2.3.1 Linear regression

In statistics, linear regression includes any approach to modeling the relationship between a dependent variable *y* and one or more independent variables denoted *x*, such that the model depends linearly on the unknown parameters to be estimated from the data. Such a model is called a "linear model". In linear regression analysis the value of a single dependent variable is predicted in means of a single independent variable and the equation resembles the equation of a linear plot;

$$y = b_1x + b_0 + a\ residual \quad \textbf{(2.1)}$$

wherein a and b are the regression coefficients and the residual is an unexplained experimental variation: x and y are the independent and dependent variables (observed value) at this selected point.

In such a case, the coefficients a and b can be predicted as follows or can be done by hand if need be;

$$b_1 = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sum (x_i - \bar{x})^2}, \quad b_0 = \bar{y} - b_1\bar{x} \quad \textbf{(2.2)}$$

The value of $\acute{y}$ (predicted value) at a certain point is found by such a regression analysis using the equation below;

$$\acute{y} = b_1x + b_0 \quad \textbf{(2.3)}$$

At a regression analysis, it is aimed to minimize the difference between $\acute{y}$ and y. In order to achieve this, the sum of squares of these residuals (SS_{res}) is attempted to be minimized. The method used is called "least square method".

$$SS_{res} = \sum (y_i - \acute{y}_i)^2 \quad \textbf{(2.4)}$$

The effectivity of these obtained regressions can be tested by ANOVA (analysis of variance) which tests if the variance is significantly reduced or this regression is a better summary of the computed data. However, the confidence interval cannot be obtained from ANOVA directly. The *f* value has to be compared to the ones for the desired confidence interval.

Another way of examining the results of our regression analysis is to calculate its coefficient of determination (correlation coefficient, R^2) which is calculated as;

$$R^2 = 1 - (SS_{res} / \text{Sum of total squares}) \quad \textbf{(2.5)}$$

If the calculated R^2 value is higher than 0.75 it is usually acceptable, while a value of 1 indicates a completely linear relationship between the predicted values according to the model and the observed experimental values [23].

2.3.2 Multiple linear regression

Although in some cases simple linear regression is sufficient to predict the response, there are many cases where this method is insufficient. In such cases, a polynomial of a single variable or more than one independent variable is needed. Multiple regression analysis is a modification of simple linear regression that enables to make regression analysis for such complex cases.

One of the biggest problems with this method is is “multicolinearity”. Multicolinearity can be defined as the case in which two or more independent variables highly correlate. This phenomena can cause misevaluation of the effects of the independent variables. Therefore studying the correlations between independent variables is crucial to obtain better results when multiple linear regression analysis is used. Another way of avoiding the multicolinearity problem is using a specially designed multiple regression which is known as response surface methodology (RSM) [23].

2.3.3 Response surface methodology

RSM is a popular and effective optimization technique for investigation of complex processes through a specially designed regression analysis. It is comprised of a group of mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables. RSM is different from fractional treatment structures as it does not only aim to determine if and how the factors (independent variable) affect the response (dependent variable) but also aims to find out the value of response in terms of factors. Hence, all variables have to be quantitive [23]. The main advantage of this method is to decrease the number of experiments which have to be conducted to determine responses at various factor levels. The results or responses at these design points (observed values) are used for a stepwise regression analysis to predict a graphic equation of the response in respect to factors in the experimental design. These graphic expressions can be either two dimensional (2-D) contour plots or three

dimensional (3-D) response surface plots. Such graphs enable a researcher to predict the response in means of independent variables at every desired point on each plot.

Different types of experimental designs can be used via RSM, but the most common type that is used is similar to fractional design. It consists of all possible combinations of low and high levels for selected variables and center points. At a center point the average values which are defined as (low + high)/2 of the variables are taken. In many cases, these center points are replicated several times for an independent experimental error approximation. These experimental levels are commonly coded as -1, 0 and +1 for minimum, center and maximum points, respectively [23,24].

A two-factor, first-order RSM experimental design is demonstrated in Figure 2.2 wherein the points represent the positions of selected experimental points and central points (0,0) [23].

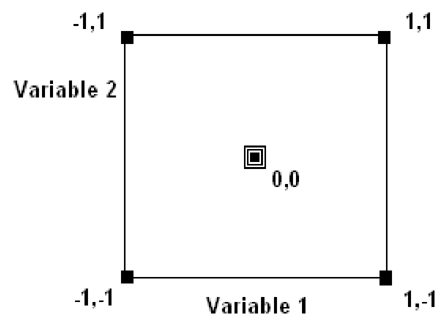

Figure 2.2: Two-factor, first-order experimental design.

The regression equation for a first-order experimental design would be:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots\ldots \beta_k x_k \qquad \textbf{(2.6)}$$

wherein β, x and y symbolize the regression coefficients, coded factor levels such as -1, 0 and +1 and the response, respectively. Such experimental designs are usually employed at the early stages of experimental research to roughly predict the appropriate variable levels. Although a first-order model has the ability to give some kind of information about the factors and the related response, it is insufficient in explaining complex relationships.

When a two-factor, second-order experimental design is applied, the regression equation includes the additional squares and cross products of the coded factor levels. The regression equation for a second-order RSM design would be:

$$
\begin{aligned}
y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots\ldots \beta_k x_k \\
+ \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \ldots\ldots \beta_{k-1} x_{k-1} x_k \\
+ \beta_{11} x_1^2 + \beta_{22} x_2^2 + \ldots\ldots \beta_{kk} x_k^2
\end{aligned}
\quad \textbf{(2.7)}
$$

The graphic representation of selected experimental design points for a two-factor, second-order RSM is shown in Figure 2.3.

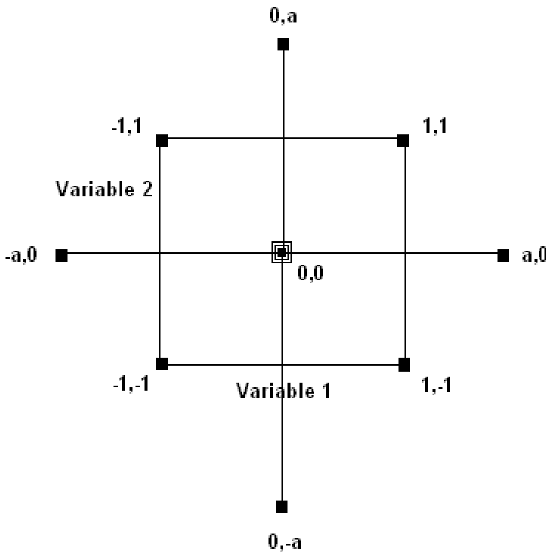

Figure 2.3: Two-factor, second-order experimental design.

The extra square and cross products in a second-order design give the resulting response surface the ability to “bend” and “flex” making it more effective by predicting complex relationships between the factors and the response.

A widely used second-order design is the central composite rotatable design wherein “axial” or “star” points are added to a first-order design. These points are generally coded with α. This α value is the distance between the center of the experimental design and the selected factor. It is calculated by the 0.25^{th} power of non-central first-order experimental design points. For instance, the value of α in Fig. 2.8 would be the 0.25^{th} power of 4 resulting in 1.414213 [23,24].

Another commonly used second-order design is the face-centered cubic design which is quite similar to the central composite rotatable design with the only difference that

the α value is replaced with 1 which results in all non-central experimental points being on the same cubic surface. Figure 2.4 displays a three-factor, second-order central composite rotatable design and face-centered cubic design [25].

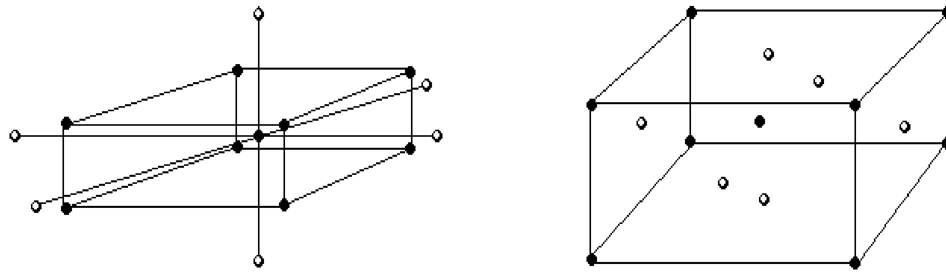

Figure 2.4: Graphic representations of three-factor, second-order rotatable central composite (left) and face-centered cubic (right) designs

3. MATERIALS AND METHODS

3.1 Materials

In this study, two processes for oil extraction of safflower seeds were performed in a laboratory scale set-up and the results were compared in terms of oil amounts (% w/w) and oil characterization: Soxhlet and aqueous enzymatic extraction (AEE). Dinçer (spineless, red colored) and Remzibey (spiny, yellow colored) safflower varieties used for the experiments were generously provided by the Ministry of Forestry and Agriculture (Konya, Turkey) around mid-September in 2009. After the removal of impurities, the safflower seeds were crushed in a coffee grinder (Sinbo), and sieved to particle sizes of <0.6 and 0.6-1 mm by using a mechanical sieve (Apparatebau Jel J. Engelsmann Akt-Gs, Germany) and stored in polyethylene bags in a refrigerator at 4 °C until needed [26].

The safflower mass was treated with two commercial enzymes, Alcalase® 2.5L and Celluclast® 1.5L, donated as brownish liquid preparation by Novozymes A/S (Bagsvaerd, Denmark) to evaluate their effects on oil extraction. Alcalase 2.5L is a highly active protease produced from a selected strain of *Bacillus licheniformis* microorganisms with a declared proteolytic activity of 2.5 AU/g (Anson Units per gram). The optimum conditions for activity are temperatures between 55–70 °C, depending on the type of substrate, and a pH range of 6.5–8.5. Celluclast 1.5L is a cellulase preparation produced from *Trichoderma reesei* microorganisms and its declared cellulolytic activity was reported to be 1.5 EGU/g (Endo-Glucanase Units per gram). It has the best activity at a temperature range of 50-60 °C and pH 4.5-6.0 [27,28]. The enzyme dosages used in extraction were based on the weight of sample and their concentration is reported as v/w, expressed as % (where 1% = 1 mL/100 g, for example) as they are provided in liquid form (v) and are applied to a solid (w) or referred to the mass of substrate. All of the enzyme complexes were refrigerated until used. Possible decline of enzyme activity was monitored by comparing yields obtained with standard AEE runs.

All chemicals used in the purification of the reaction products, their analyses and as solvents were purchased from Merck Chemical Co. (Darmstadt, Germany). These reagents were of analytical grade and the highest purity needed for each application. Deionized water was used in all experiments [26].

3.2 Methods

Ground seeds that were screened to different particle sizes served for the characterization and oil extraction of safflower seeds. Pre-treatments such as homogenization, ultrasonication [29], mechanical treatments (extruding and flaking) [28, 30], incubation in boiling water bath (hydrothermal pre-treatment) [31], or oven drying the seeds at 105 °C steps were not applied to the seeds prior to extraction in order to improve oil recovery. All experiments were replicated at least twice for both particle sizes, with duplicate samples (using two types of enzymes) for each experiment in AEE. The mean values of data were reported as the mean±SD (standard deviation) and the SD for our results never exceeded more than 3%.

3.2.1 Characterization of safflower seeds

The average weight, size and hull amount of the safflower seeds were calculated by using 250 seeds. The moisture content was determined as the loss of weight at 105 °C for 3 h. Total oil was extracted by Soxhlet method using food grade *n*-hexane. All analytical determinations were performed according to the methods of AOCS standards [32].

3.2.2 Oil extraction from safflower seeds

The oil content of safflower seeds was determined by the Soxhlet extraction process. For this assay, 25 g of ground seed material was fed to a Soxhlet extractor fitted with a 0.5 L round-bottom flask and a condenser. The extraction was carried out first for 4 h then for a further 2 h with 0.3 L of n-hexane in a heat jacket. After extraction, the solvent was distilled off under a vacuum using a rotary evaporator (Laborata 4000-efficient Heidolph, USA) at 70 °C for safflower oil recovery and the oil obtained was weighed and stored under refrigeration at 4 °C, until used for further analyses.

As for the solid-liquid extraction at room temperature, 35 g of ground safflower seeds were weighed into a 0.8 L glass beaker, and 0.5 L of n-hexane was added. The

mixture was stirred for 2 h at room temperature on a magnetic stirrer. During the mixing process, the light and heat factors were minimized. Finally, the slurry was filtered through a funnel and hexane phase containing safflower oil was obtained as the filtrate. The solvent was evaporated under a vacuum using the rotary evaporator for safflower oil recovery and the oil obtained was weighed [32].

3.2.3 Aqueous enzymatic oil extraction

Ground safflower seeds were subjected to a hydrolytic treatment with cellulase and protease during aqueous processing of oil extraction. The AEE process investigated in this research was a slightly adapted version of an earlier method reported by Moreau *et al*. [33]. A standard process was established after preliminary experiments: 3 g samples of ground safflower seeds were weighed into 50 mL polycarbonate centrifuge tubes and suspended with 20 mL of buffer solution (KH_2PO_4 and $NaH_2PO_4.H_2O$) giving a mixture at a 1:7 (w/v) ratio (diluion ratio), pH 5. At this pH value, safflower proteins are insoluble and can be recovered as a concentrate in the solid phase, removing oil and phenolic compounds in the liquid one. Optimum pH for enzymes used in this study is in the range of 4.5–8.5. Thus, the pH was the unmodified pH of the aqueous suspension of the mixture and kept constant throughout the extraction period. 0.5 mL of each Alcalase 2.5L and Celluclast 1.5L was added separately and the mixture was incubated. The hydrolytic enzymatic treatment to enhance oil extractibility was performed during the churning stage (incubation stage), which was carried out in an orbital incubator (Edmund Bühler, KS-15, Germany) with tubes shaking horizontally at 200 rpm and 50 ºC (the optimum to preserve the quality of the products, and to favor both the activity and stability of enzymes) for 6 h. Following the incubation, the tubes were cooled at room temperature for 20 min and suspension was centrifuged at 4000 rpm for 30 min using a swinging basket type centrifuge (Universal 32, Hettich Zentrifugen, Germany) resulting in four phases: recovered free oil, creamy phase, aqueous phase and a pellet of insoluble material. The main difficulty of this study was the formation of an emulsion after centrifugation in all reaction conditions investigated [26]. Hence, the oil was recovered by using various separation methods such as using a micropipette, vacuum filtration, and separation funnel filtration in order to reach the highest oil yield.

Using a Micropipette: by using a Pasteur pipette, the top, oil-rich phase was carefully collected, followed by the creamy and aqueous phase, leaving the meal at the bottom and the oil obtained was weighed [34].

Vacuum Filtration: the mixture, leaving the meal at the bottom of the tube, was decanted into a porcelain Buchner funnel and allowed to filter by vacuum power. Then, the emulsion formed in the nuche erlenmayer was poured into a separating funnel and allowed to separate into the oil-bearing hexane phase and water layer. The water layer was then drained off to obtain the oil. The solvent was evaporated under vacuum power by the rotary evaporator and the oil obtained was weighed.

Separation Funnel Filtration: the emulsion, leaving the insoluble pellet at the bottom of the tube, was decanted into a separating funnel and allowed to separate into the oil bearing hexane phase and water layer. The water layer was then drained off to obtain the oil. The solvent was evaporated under vacuum by the rotary evaporator and the oil obtained was weighed.

3.2.3.1 Selection of independent variables

For experimental design, preliminary experiments were established mainly to evaluate the feasibility of enzymatic treatment of safflower seeds and to select the operational range of the most important and effective variables. Several assays using the more suitable formulations found in previous reports were implemented. In this study, sub-optimal extraction conditions including enzyme type and amount, (0.5 to 3.0 mL enzyme⁄g substrate), buffer solution pH (4 to 8), incubation time (4 to 8 h) and temperatures (30 to 60 °C), churning rate (100 to 400 rpm), ratios of seed to buffer solution (1:3, 1:5, 1:7, 1:9 w⁄v), particle size (<0.6 and 0.6-1 mm), cenrifugation conditions and separation methods were varied to determine their influence on the oil yield. The operational variables were independently varied, keeping other parameters fixed, so as to obtain the best conditions for extraction. The criterion for selecting operating conditions was the oil extractability [35].

The oil amount (% w⁄w) is the direct weight measurement of the oil obtained from the aqueous enzymatic extraction after centrifugation. To compare the oil extractability under different reaction conditions, the oil yield (% w⁄w) calculated was based on the initial oil content in safflower seeds with the same particle size as

determined by the Soxhlet method and the oil amount. The oil amount and oil yield were expressed as follows:

Oil amount (% w/w) = oil extracted by AEE

Oil yield (% w/w) = (oil extracted by AEE / total oil extracted by *Soxhlet* extraction) x 100

3.2.3.2 Experimental design and statistical analysis of experimental data

Response surface methodology (RSM) was employed to optimize the enzymatic process parameters. In this research, the combined effect of enzyme amount, buffer solution pH, and incubation temperature on oil amount was evaluated by this method. A three-factor, three-level cubic central composite design requiring a total of 17 experiments with 3 centre points (0, 0, 0) was computed. The three factors selected were incubation temperature (X_1, °C), enzyme amount (X_2, mL enzyme/g substrate) and buffer solution pH (X_3). The response was the oil amount (Y, % w/w). Before RSM was applied, these approximate conditions were examined by varying one independent variable at a time while keeping the others constant: an appropriate range for each independent variable was determined. Based on the preliminary experiments, the ranges of settings for the three independent variables were chosen as follows: incubation temperature, 40-60 °C; enzyme amount, 0.5-2.5 mL enzyme/3 g substrate; buffer solution pH, 4-6.

The obtained data were computed by using the statistical software (Statistica 7.0 Software, StatSoft Inc., Tulsa, OK, USA) for optimization of these three most effective parameters through RSM. Statistical analysis of the experimental data was performed by analysis of variance (ANOVA) to fit the second-order polynomial model of the following form predicted for the optimization:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad \textbf{(3.1)}$$

This model shows individual and crossed effects of each variable where Y is the response (oil amount, % w/w) which represents the estimated dependent variable; X_1, X_2 and X_3 are the levels of the coded independent variables; β_0 is the intercept term; β_1, β_2 and β_3 are the linear terms; β_{12}, β_{13} and β_{23} are the interaction terms; β_{11}, β_{22} and β_{33} are the quadratic terms of the factors. Contour and response surface plots

were obtained by keeping the least effective independent variable at a constant value while changing the other two independent variables. *P* values indicate the confidence of the calculated values. A probability value at $p<0.05$ was considered statistically significant and *p* values less than 0.01 indicate a higher confidence interval of 99%. To confirm the optimum conditions of aqueous extraction assisted by Celluclast 1.5L, oil extractions were carried out under the determined optimum conditions [27,35,36].

3.2.4 Characterization of safflower oil

The physicochemical properties of oils extracted from safflower seed with the same particle size by the Soxhlet extraction and aqueous enzymatic extraction were compared in terms of their acid value (AV, mg KOH⁄g oil), saponification value (SV, mg KOH⁄g oil), refractive index (RI), free fatty acids composition (FFA, %) and unsaponified matter (UM, % w⁄w) using standard analytical methods.

To determine the FFA composition of the safflower oil, they were first converted to their corresponding methyl esters (FAMEs) by the BF_3⁄methanol esterification procedure [32]. The fatty acid composition of the FAMEs was then analysed using HP 5890 II gas chromotograph (Hewlett Packard, Waldron, Germany) equipped with a HP- INNOWAX capillary column (30 m x 0.32 mm x 0.5 µm film thickness of polyethylene glycol (PEG); Teknokroma, Barcelona, Spain) and a flame ionisation detector (FID). The detector temperature was 280 ºC and the injection temperature was 250 ºC. Oven temperature was kept constant at 150 ºC for 5 min, raised from 150 to 275 ºC for 10 min. Oxygen-free nitrogen was used as carrier gas at a flow rate of 1.6 mL min^{-1}. The flow rates of hydrogen and air were 33 and 460 mL min^{-1}, respectively. A sample volume of 0.6 µL was injected using split mode with a split ratio of 1:88. Peaks were identified by comparing their retention times with those of a mixture of standard methyl esters analyzed at the same conditions and their areas were integrated with an integrator. The FFA composition was reported as the relative percentage of the total peak area.

4. RESULTS AND DISCUSSION

4.1 Characterization of Safflower Seeds

The moisture and oil contents, average weight, average size, and hull amounts of safflower seeds are shown in Table 4.1 along with some other safflower varieties from the literature.

Table 4.1: Properties of some safflower varieties [37]

Safflower varieties	Average weight 10^{-5} (kg)	Average size 10^{-3} (m)	Av. hull content (% w/w)	Moisture content (% w/w)	Oil content (% w/w)	
					<0.6 mm	0.6-1 mm
Remzibey	3.7	4.8	45	4.9±1.1	43.7±1.3 [a]	38.3±0.6[a];29.6±0.8[b]
Dinçer	3.9	6.3	52	5.6±0.9	41.8±1.2 [a]	36.1±0.9[a];27.4±1.0[b]
Yenice*	4.4	7.4	40	7.8	-	32.8[a]
614.1*	6.2	7.4	46	7.7	-	29.8[a]

Values are calculated as percentage on original seed weight basis for safflower seed samples
[a] Oil amount by Soxhlet extraction
[b] Oil amount by Solid-liquid extraction at room temperature

According to the table, some properties of safflower seeds were identified but similar results were obtained. Işığıgür [37] noted 28.9% oil content, 8.1% moisture content, 46% hull amount, 6.61×10^{-3} (m) seed size and 3.8×10^{-5} (kg) weight for the Dinçer variety, close to the results obtained in this present study. Variations in oil content and other properties may be due to differences in the variety of plants, growth climate, ripening stage, the harvesting period of the seeds, and extraction conditions [27]. Dinçer variety was chosen for aqueous enzymatic oil extraction of safflower because the properties of each safflower seed are similar.

4.2 Selection of Independent Variables and Experimental Design

4.2.1 Centrifugation conditions (rpm, min)

The centrifugation conditions were optimized, wherein the reaction products were centrifuged in the range from 2500 to 4000 rpm for 20 to 60 min for separation of solid and liquid phases. When the centrifugation time was fixed at 60 min, the oil amount increased with the centrifugation speed until a maximum was obtained at 4000 rpm. When the centrifugation time was shortened to 30 min at 4000 rpm, it was found that this shorter time was sufficient to float the free oil and that additional centrifugation time did not increase oil amounts. The best results were obtained at 4000 rpm and the use of longer centrifugation times did not offer better results than the level used in this research (data not shown) [38].

4.2.2 Particle size (mm) and incubation time (h)

The effects of the processing parameters such as enzyme type, particle size, pH, and incubation time on the oil amount extracted by using different separation methods were studied and compared. The incubation time and particle size would affect the economy of the enzymatic treatment, so that an evaluation of the impact of these operational variables is fundamental. For these assays, the suspension of ground safflower seeds (3 g in 20 ml buffer solution, pH 4, 5, 6) were mixed with 0.5 mL of each Alcalase 2.5L and Celluclast 1.5L separately and incubated for a period ranging from 4 to 8 h at 50 °C with constant shaking at 200 rpm. After centrifugation, the oil was obtained by using various separation methods.

In the case of particle size, the degree of particle size reduction is a determining factor in biohydrolytic reactions. The amount of extracted oil depends on particle size and after 6 h of Soxhlet extraction of ground samples nearly all the oil present was extracted (Table 4.1). The favourable effect of a size reduction on the oil amount can be observed in Figure 4.1, wherein the amount of oil extracted increased by 3.5% at pH 5 and 6 h incubation time when particle size reduced from 0.6-1 to <0.6 mm.

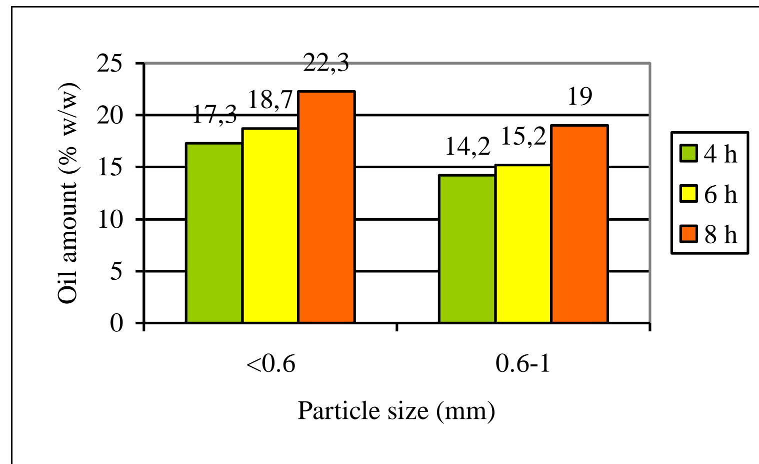

Figure 4.1: Oil recoveries from safflower in function of particle size and incubation time by Pasteur pipette: buffer solution pH, 5; enzyme amount 0.5 mL Alcalase 2.5L/3 g substrate; dilution ratio 1:7 (w/v); churning rate, 200 rpm; incubation temperature, 50 °C.

In the case of Celluclast 1.5L, it can be also observed in Figure 4.2 that the amount of oil extracted increased by almost 4% at pH 5 and 6 h incubation time when particle size reduced from 0.6-1 to <0.6 mm.

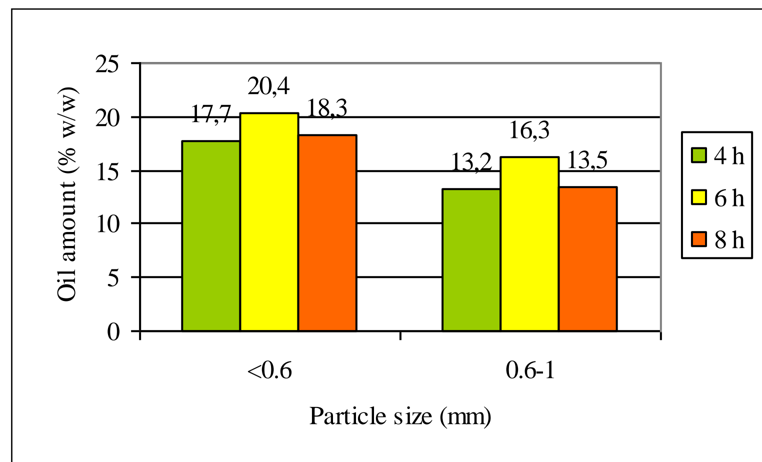

Figure 4.2: Oil recoveries from safflower in function of particle size and incubation time by Pasteur pipette: buffer solution pH, 5; enzyme amount 0.5 mL Celluclast 1.5L/3 g substrate; dilution ratio 1:7 (w/v); churning rate, 200 rpm; incubation temperature, 50 °C.

In this present work, best results are obtained with the <0.6 mm particle size which has separation problems compared to 0.6-1 mm particle size but it is more homogeneous in terms of the seed hulls. Evaluating these results, the particle size was selected as <0.6 mm for the enzymatic treatment in this research. As expected, a reduction in particle size means more interaction surface for enzymes, therefore

allowing a better accessibility of enzyme to the cell wall [39]. Santamaria *et al.* [26] also observed that highest extraction yield was obtained by using seeds ground to a particle size of approximately 0.4–0.6 mm diameter.

As for the treatment time, for Alcalase 2.5L, when incubation time was increased from 4 to 6 h the oil amount improved by about 1% at pH 4, 2% at pH 5 and 3% at pH 6 for small particle size, whereas a further increase to 8 h caused an additional increase of only 4% at pH 4, 4% at pH 5 and 6% at pH 6. Although a long incubation time (8 h) favors oil extractibility more than an intermediate time (6 h), 6 h is long enough to carry out enzymatic treatment efficiently. As there is not a noticeable difference between the amount of oil obtained when the extraction time was increased from 6 h to 8 h, 6 h was chosen as an optimum time for the enzyme-aided aqueous process (Figure 4.3).

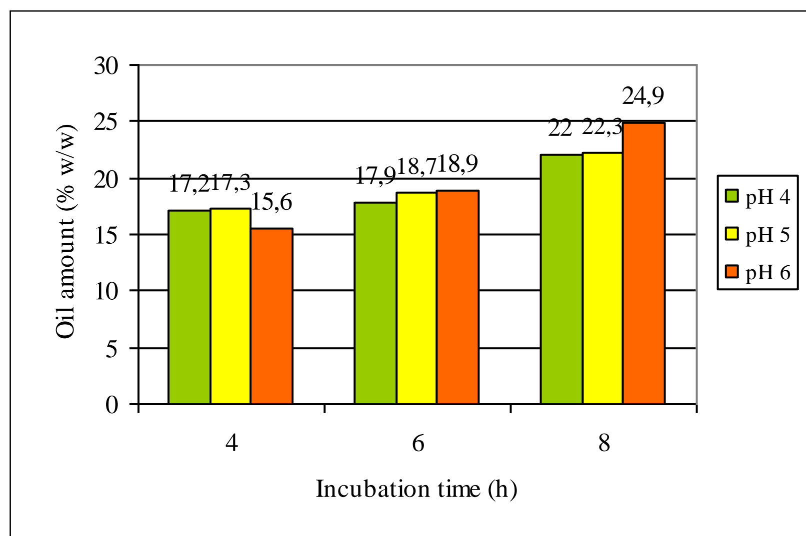

Figure 4.3: Oil recoveries from safflower in function of incubation time and pH by Pasteur pipette: enzyme amount, 0.5 mL Alcalase 2.5L/3 g substrate; dilution ratio, 1:7 (w/v); particle size, <0.6 mm; churning rate, 200 rpm; temperature, 50 °C.

When Celluclast 1.5L was used, increasing the incubation time from 4 h to 6 h increased the oil amount by about 2% at pH 4, 3% at pH 5 and 1% at pH 6 whereas increasing the incubation time from 6 h to 8 h decreased the oil amount by about 1% at pH 4, 2% at pH 5 and less than 1% at pH 6 using small particles. Since long incubation time (8 h) decreased the extractibility of oil, 6 h was chosen to be an optimum for the enzymatic treatment assisted by Celluclast 1.5L (Figure 4.4).

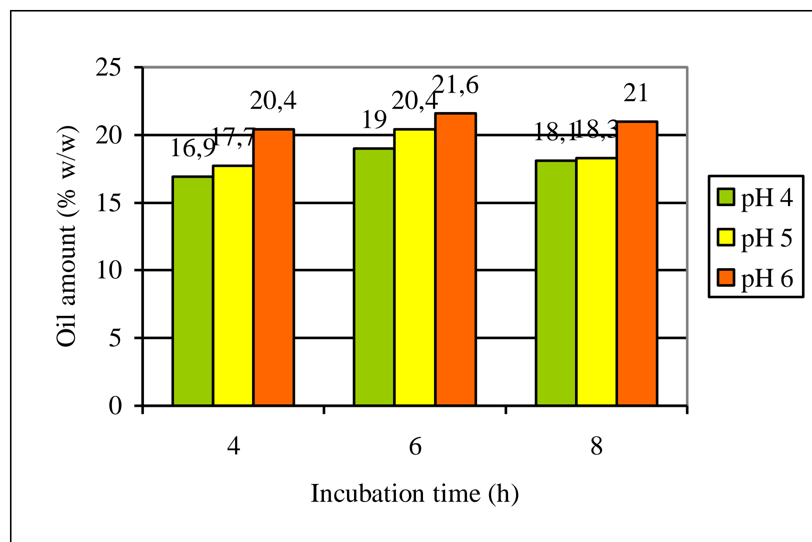

Figure 4.4: Oil recoveries from safflower in function of incubation time and pH by Pasteur pipette: enzyme amount, 0.5 mL Celluclast 1.5L/3 g substrate; dilution ratio, 1:7 (w/v); particle size, <0.6 mm; churning rate, 200 rpm; temperature, 50 °C.

Hence, incubation times were fixed at 6 h according to the results of these preliminary tests and adopted for the rest of the experiments. The results obtained are in agreement with the findings of Ramadan *et al.*[39] who reported that the effects of the enzymatic treatment are completed in a period of 2 h although slight increments can be obtained in more extended periods. Dominguez *et al.* [14] also noted that in the case of soybean oil extraction, the hydrolysis time also had a strong effect on oil yield, with a period of 6 h reported to be suitable.

Regarding separation methods, initially, the top oil layer was collected by using a Pasteur pipette. However, because of the formation of a white emulsion layer, difficulty was encountered in separating the oil from the particles that were suspended in the emulsion. After that, the vacuum filtration method which had the problem of filtering the emulsion and oil loss was used to recover the oil. It was suggested that the blockage in the pores might be something to do with the hydrolysis products of protein and cellulose. Finally, the separation funnel method was suggested and all experiments were conducted according to this method.

The effects of the process parameters such as enzyme type, particle size, pH, and incubation time on the oil amount extracted by using different separation methods are displayed in Table 4.2.

Table 4.2: Effect of particle size, buffer solution pH and incubation time on the oil yield using various separation methods

Enzyme type	Particle size (mm)	pH	Incubation time (h)	Oil amount (% w/w)		Oil yield (% w/w)	
				Pasteur pipette	Vacuum Filtration	Pasteur pipette	Vacuum filtration
Alcalse 2.5L	< 0.6	4	4	17.2±0.3	27.2±1.7	40.9	65.0
			6	17.9±0.2	30.0±1.8	42.8	71.8
			8	22.0±0.8	31.2±1.4	52.6	74.6
		5	4	17.3±0.0	28.4±1.5	41.4	67.9
			6	18.7±1.1	30.9±1.5	44.7	73.9
			8	22.3±0.9	31.9±1.7	53.4	76.3
		6	4	15.6±0.7	29.0±0.8	42.1	69.4
			6	18.9±0.7	31.0±1.2	45.2	74.2
			8	24.9±1.0	32.9±1.3	59.6	78.7
	0.6-1	4	4	13.9±0.6	18.5±1.2	38.5	51.3
			6	15.1±0.5	20.3±1.3	41.8	56.2
			8	17.5±0.0	23.1±1.0	48.5	64.0
		5	4	14.2±0.6	19.2±0.4	39.0	53.2
			6	15.2±0.7	21.4±0.5	42.6	59.3
			8	19.0±1.0	23.9±1.2	52.6	66.2
		6	4	14.5±0.8	19.9±1.3	40.2	52.9
			6	15.9±0.8	23.6±1.2	44.0	65.4
			8	21.1±1.4	24.8±0.9	58.5	68.7
Celluclast 1.5L	<0.6	4	4	16.9±1.1	-	40.4	-
			6	19.0±0.9	29.0	41.8	69.4
			8	18.1±0.7	-	43.3	-
		5	4	17.7±0.7	-	42.3	-
			6	20.4±0.4	28.4	48.8	67.9
			8	18.3±0.3	-	43.8	-
		6	4	20.4±1.0	-	48.8	-
			6	21.6±0.8	29.4	51.7	70.3
			8	21.0±1.1	-	50.2	-
	0.6-1	4	4	12.8±0.8	-	35.5	-
			6	14.5±0.5	15.0	40.2	41.6
			8	13.8±0.7	-	38.2	-
		5	4	13.2±0.9	-	36.6	-
			6	16.3±1.0	16.2	45.2	44.9
			8	13.2±1.3	-	36.6	-
		6	4	13.5±1.6	-	37.4	-
			6	17.9±0.8	13.4	49.6	37.1
			8	12.6±0.9	-	34.9	-

4.2.3 Churning rate (rpm)

The control of the churning rate during extraction is fairly critical in terms of its influence on the oil yield. The level of shaking speed required for optimal recovery was determined by using selected levels of 100, 200, 300 and 400 rpm for both 0.5 mL of each Alcalase 2.5L and Celluclast 1.5L. Slurry was made of 3 g safflower seeds (<0.6 mm) in 20 mL buffer solution, pH 5, 6 h incubation time at 50 °C.

Table 4.3: Impact of churning rate on the oil recovery

Enzyme type	Oil amount (% w/w)			
	100 rpm	200 rpm	300 rpm	400 rpm
Alcalase 2.5L	26.3±0.6	27.9±0.5	24.3±1.0	18.8±1.1
Celluclast 1.5L	18.0±1.4	19.7±0.8	16.8±0.7	13.2±1.2

Table 4.3 shows the impact of churning rate on oil recovery, the obvious maximum being at 200 rpm for both Alcalase 2.5L and Celluclast 1.5L. Oil amount increased with churning rates from 100 to 200 rpm, but it began to fall again when the churning rate rose further from 200 to 400 (Figure 4.5). The reasons for this effect may be because of high shaking speed which would not favor oil-in-water emulsion formation which is the principle of separation and emulsion stability. As 200 rpm provided the best oil extractibility, this churning rate was chosen as the optimal value and employed in all of the rest of the experiments. Also, it was observed that during the experiments, there was much less free oil on top at higher churning levels. The results obtained are in agreement with the findings of Sharma *et al.* [40] who reported that a decrease in shaking speed led to a decrease in oil recovery. Increasing the speed led to emulsification and reduced the amount of clear oil obtained at the top.

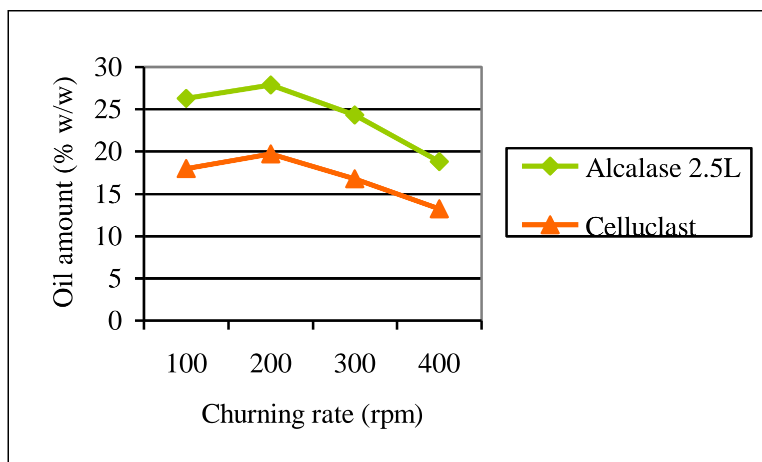

Figure 4.5: Effect of churning rate on the oil recovery: incubation time , 6h; enzyme amount, 0.5 mL Alcalase 2.5L/3 g substrate; 0.5 mL Celluclast 1.5L/3 g substrate buffer solution pH, 5; dilution ratio, 1:7 (w/v); particle size, <0.6 mm; temperature, 50 °C.

4.2.4 Enzyme amount (mL enzyme/g substrate)

The cost of enzymes is one of the major economic factors of the process, so that the enzyme amount has to be optimized during the treatment. It was, therefore, fundamental to determine whether a higher enzyme amount could provide more efficient oil yields. The level of enzymes required for optimal recovery was determined by using enzyme levels of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL for both Alcalase 2.5L and Celluclast 1.5L. Slurry was made of 3 g safflower seeds (<0.6 mm) in 20 mL buffer solution, pH 5, 6 h incubation at 50 °C and 200 rpm. The effect of enzyme amount on the amount of oil extracted is demonstrated in Table 4.4.

Table 4.4: Effect of enzyme amount on the oil recovery

Enzyme type	Oil amount (% w/w)					
	0.5 mL	1.0 mL	1.5 mL	2.0 mL	2.5 mL	3.0 mL
Alcalase 2.5L	27.9±0.5	29.9±0.7	31.1±1.3	31.5±0.7	31.4±1.0	31.3±0.8
Celluclast 1.5L	19.7±0.8	25.3±1.0	28.7±0.8	31.1±1.00	32.6±0.9	32.8±0.6

Table 4.4 indicates that an increase of the enzyme amount generally increased oil amount. Drastic increases in the oil amount occurred as the enzyme amount was raised from 0.5 to 1.5 mL and 0.5 to 2.5 mL when Alcalase 2.5L and Celluclast 1.5L was used, respectively. The total effect on the oil extractibility remains almost

unaffected when these enzyme amounts are raised from 1.5 to 2.0 for Alcalase 2.5L and from 2.5 to 3.0 for Celluclast 1.5L. However, the maximum oil amount of about 33% was obtained with a 3.0 mL of Celluclast 1.5L and 31% oil amount when 2.0 mL of Alcalase 2.5 L was used (Figure 4.6).

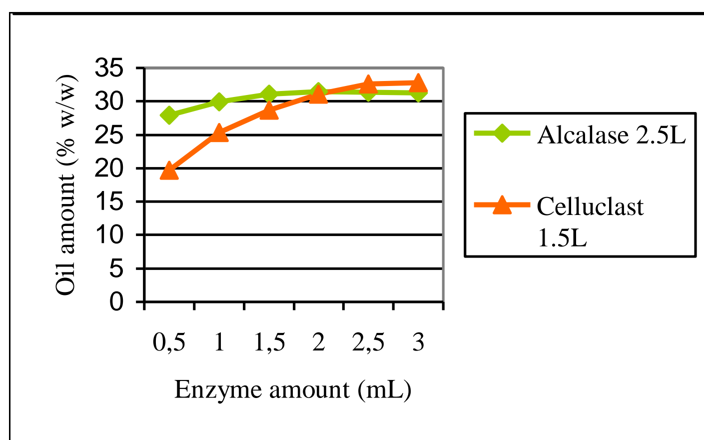

Figure 4.6: Effect of enzyme amount on the oil recovery: incubation time , 6h; buffer solution pH, 5; dilution ratio, 1:7 (w⁄v); particle size, <0.6 mm; churning rate, 200 rpm; temperature, 50 °C.

Ramadan *et al.*[39] repoted that an additional increase of enzyme amount does not mean a parallel improvement in oil amount, but a rise in expense which is a disadvantage from the economic point of view. The analysis of these results show that the enzyme amount ought to be a compromise between the improvement in the extractibility and the cost of the enzyme. As 1.5 mL Alcalase 2.5L⁄3 g substrate and 2.5 mL of Celluclast 1.5L⁄3 g substratee in 20 mL slurry was satisfactory for enzymes evaluated in this study, this amount was adopted in all subsequent experiments.

4.2.5 Incubation temperature (°C)

The dependence of the process on the temperature is an important factor as it would affect the cost of the enzymatic treatment so the incubation temperature has to be optimized. It was therefore, important to investigate whether a higher temperature could provide a more efficient oil recovery. Safflower seeds (<0.6 mm) were treated for a period of 6 h at 200 rpm with Alcalase 2.5L (1.5 mL) and Celluclast 1.5L (2.5 mL) diluted to a ratio of 3 g sample⁄20 mL buffer solution at pH 5 and temperatures of 30, 40, 50 and 60 °C. Table 4.5 shows how enzymes affect the oil amount at different temperatures.

Table 4.5: Impact of incubation temperature on the oil recovery

Enzyme type	Oil amount (% w/w)			
	30 °C	40 °C	50 °C	60 °C
Alcalase 2.5L	30.5±0.3	32.1±0.2	31.1±1.5	26.0±0.8
Celluclast 1.5L	17.9±0.1	30.7±0.3	32.6±0.9	15.7±0.4

When the reaction temperature was raised from 30 to 40 °C the oil amount increased from 31 to 32% and from 18 to 31% for Alcalase 2.5L and Celluclast 1.5L, respectively. Raising the temperature to 60 ^{0}C led to a reduced oil amount of 26% for Alcalase 2.5L and 16% for Celluclast 1.5L (Figure 4.7). Jovanovic *et al*. [38] and Sharma *et al.* [40] have pointed out that this is presumably because the enzymes become thermo-inactivated.

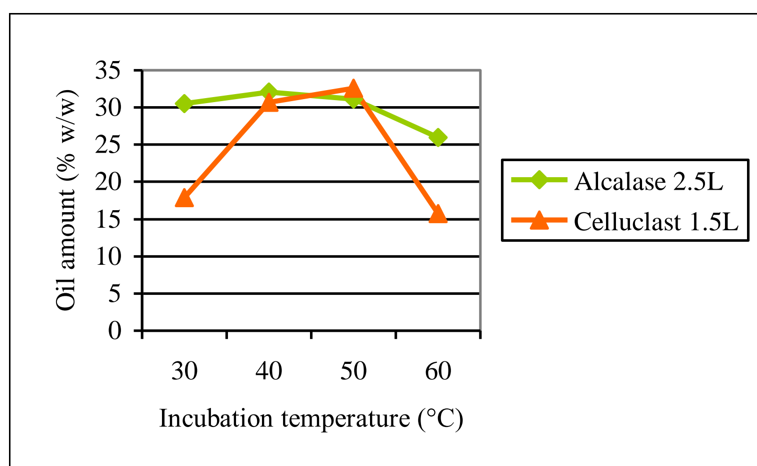

Figure 4.7: Effect of incubation temperature on the oil recovery: incubation time, 6h; enzyme amount, 1.5 mL Alcalase 2.5L/3 g substrate and 2.5 mL Celluclast 1.5L/3 g substrate buffer solution pH, 5; dilution ratio, 1:7 (w/v); particle size, <0.6 mm; churning rate, 200 rpm.

As a result, lower temperatures result in lower extraction yields, but higher temperatures affect enzyme stability. The temperature of 40 °C which is in the optimal temperature range of Celluclast 1.5L was chosen in this present research and this temperature was employed for the rest of the experiments.

4.2.6 Buffer solution pH

The effect of pH on oil amount using both enzymes were examined in the region where commercial enzymes are reportedly stable. 3 g of ground safflower (<0.6 mm) were dispersed in 20 ml buffer solution at pH values ranging from 4 to 8 and stirred to make a suspension. To this 1.5 mL of Alcalase 2.5L and 2.5 mL of Celluclast 1.5L were added and incubated for 6 h at 40 °C with constant shaking at 200 rpm. Table 4.6 shows the effect of Alcalase 2.5L and Celluclast 1.5L at five different pH values.

Table 4.6: Effect of buffer solution pH on the oil recovery

Enzyme type	Oil amount (% w/w)				
	pH 4	pH 5	pH 6	pH 7	pH 8
Alcalase 2.5L	31.8±1.1	32.1±1.0	33.4±0.7	31.5±0.8	30.3±0.4
Celluclast 1.5L	27.3±1.2	30.7±0.8	22.5±1.3	19.3±0.8	14.1±1.5

As for Alcalase 2.5L, when the pH increased from 4 to 5, oil amount rose slightly. The maximum amount of 33.4% oil was obtained at an extraction pH of 6, with lower but not significantly different oil amounts in the range of pH 7 to 8. When Celluclast 1.5L was used, increasing the pH from 4 to 5 resulted in the maximum amount of 31% oil at an extraction pH of 5, with lower but not significantly different oil amounts in the range of pH 6 to 8 (Figure 4.8).

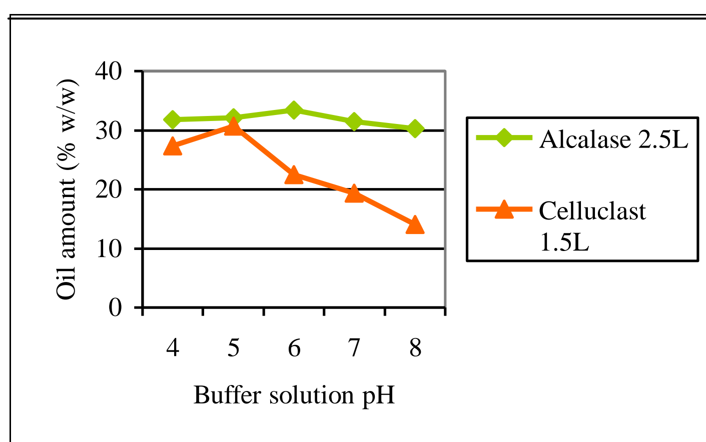

Figure 4.8: Effect of buffer solution pH on the oil amount: incubation time, 6h; enzyme amount, 1.5 mL Alcalase 2.5L/3 g substrate and 2.5 mL Celluclast 1.5L/3 g substrate; dilution ratio, 1:7 (w/v); particle size, <0.6 mm; churning rate,200 rpm; temperature, 40 °C.

4.2.7 Seed to buffer solution ratio (g substrate/mL buffer solution)

Water plays an important role in AEE processes by favoring wall degradation as well as diffusion and mobility of both enzymes and the substrate. The subsequent drying of meal after enzymatic reaction is one of the major expenses of the process so sample-to-buffer solution (dilution) ratio should also be optimized. To determine the effect of the seed-to-buffer solution ratio on the AEE process, several experiments assisted by Alcalase 2.5L and Celluclast 1.5L at sample-to-buffer solution ratios ranging from 1:3 to 1:9 (w/v) were conducted at pH 5 and 40 °C. An enzyme amount of 1.5 mL Alcalase 2.5L/3g sample and 2.5 mL Celluclast 1.5L/3 g sample (<0.6 mm) and 6 h treatment time at 200 rpm were employed. Table 4.7 displays the results obtained.

Table 4.7: Impact of seed-to-buffer solution ratio on the oil recovery

Enzyme type	Oil amount (% w/w)			
	1:3	1:5	1:7	1:9
Alcalase 2.5L	28.6±1.1	29.9±1.2	32.6±1.4	29.5±0.7
Celluclast 1.5L	22.6±1.3	24.5±1.1	30.9±1.6	19.8±0.5

As regards the influence of seed-to-buffer solution ratio on oil extractibility, the apparent maximum is in the range of (1 g sample/5 mL buffer to 1 g sample/7 mL buffer solution). The oil amount increased with dilution ratios (from 1:3 to 1:5), but it began to fall again when the substrate became more diluted (from 1:7 to 1:9) (Figure 4.9). Ramadan *et al.* [38] reported that the reasons for this effect may be due to both the acidic pH which would not favor oil-in-water emulsion formation which is the principle of separation and emulsion stability. Furthermore, an increase in dilution ratios means low accessibility of enzymes to cell walls. As a 1 g sample/7 mL buffer ratio provided a better oil extractibility, this dilution ratio was selected as optimum and employed in all subsequent experiments.

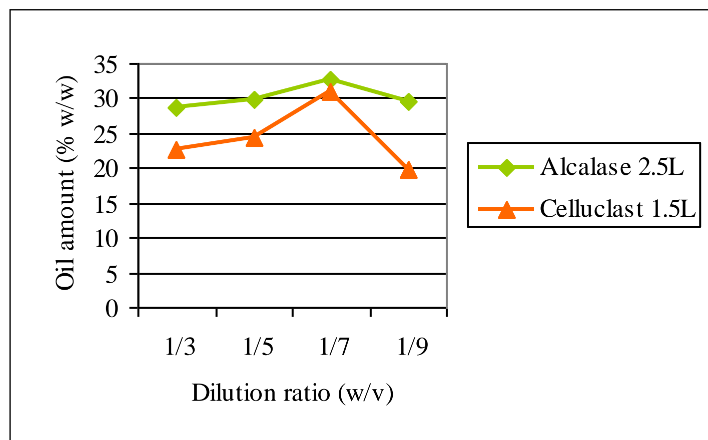

Figure 4.9: Effect of dilution ratio on the oil amount: incubation time, 6h; enzyme amount, 1.5 mL Alcalase 2.5L⁄3 g substrate and 2.5 mL Celluclast 1.5L⁄3 g substrate; buffer solution pH,5; particle size, <0.6 mm; churning rate,200 rpm; temperature, 40 °C.

Although, aqueous enzymatic treatment assisted by Alcalase 2.5L resulted in significantly higher oil amounts, Celluclast 1.5L was chosen for the optimization since the effects of the operational variables on the oil amount were more drastic when Celluclast 1.5L was used (Alcalase 2.5L released more free oil at the top). Hanmoungjai *et al.* [17] investigated the extraction of oil from rice bran using commercial enzymes. It was found that the oil yield obtained by using commercial protease (Alcalase) was higher than those obtained by using other enzymes.

After preliminary evaluation and literature research, the parameters that needed to be optimized and their corresponding levels as +1, 0 and -1 were determined. The center point for each independent variable is coded 0. The highest and lowest levels of the independent variables are coded +1 and -1, respectively. The real and coded levels of the independent variables are displayed in Table 4.8.

Table 4.8: Coded and real levels of the independent variables used in the design

Independent variables	Factor level		
	-1	0	+1
Incubation temperature (X_1, °C)	40	50	60
Enzyme amount (X_2, mL enzyme⁄g substrate)	0.5	1.5	2.5
Buffer solution pH (X_3)	4	5	6

The 17 designed experimental points and their actual and coded levels and the observed responses are shown in Table 4.9. The maximum oil amount of 33.3% was observed at a temperature of 50 °C, an enzyme amount of 2.5 mL and a buffer solution pH of 4 (run 7).

Table 4.9: Three-factor, three-level cubic central composite design and responses for aqueous enzymatic oil extraction

Run	Temperature (^0C) (X_1)	Enzyme amount (mL) (X_2)	Buffer solution PH (X_3)	Observed oil amount (% w/w) (Y)	Predicted oil amount (% w/w)	Residual value
1	-1 (30)	-1 (0.5)	-1 (4)	17.9	17.6	0.3
2	-1 (30)	-1 (0.5)	+1 (6)	17.7	18.3	-0.6
3	-1 (30)	+1(2.5)	-1 (4)	18.9	19.8	-0.9
4	-1 (30)	+1(2.5)	+1 (6)	17.4	16.9	0.5
5	+1 (50)	-1 (0.5)	-1 (4)	24.4	24.9	-0.5
6	+1 (50)	-1 (0.5)	+1 (6)	23.0	22.2	0.8
7	+1 (50)	+1(2.5)	-1 (4)	33.3	32.7	0.6
8	+1 (50)	+1(2.5)	+1 (6)	26.2	26.5	-0.3
9	-1 (30)	0 (1.5)	0 (5)	21.6	20.7	0.9
10	+1 (50)	0 (1.5)	0 (5)	28.7	29.2	-0.5
11	0 (40)	-1 (0.5)	0 (5)	27.4	27.2	0.2
12	0 (40)	+1 (2.5)	0 (5)	30.7	30.5	0.2
13	0 (40)	0 (1.5)	-1 (4)	24.5	23.8	0.7
14	0 (40)	0 (1.5)	+1 (6)	20.8	21.1	-0.3
15	0 (40)	0 (1.5)	0 (5)	27.0	26.9	0.1
16	0 (40)	0 (1.5)	0 (5)	26.3	26.9	-0.6
17	0 (40)	0 (1.5)	0 (5)	26.7	26.9	-0.2

4.3 Statistical Analysis of Experimental Data

Depending on the interactions between dependant (Y) and independent variables (X_1, X_2 and X_3) their linear, quadratic and interactive coefficients (regression coefficients) were determined and represented in Table 4.10 along with *p* values. Regression coefficients are obtained by using a least squares technique to predict a quadratic polynomial model for response (oil amount, % w/w). In this case, 8 effects have *p* values less than 0.05 and 7 of these have $p<0.01$which indicates a higher confidence interval of 99%.

Determination of the optimized conditions of Celluclast 1.5L resulted in an oil amount model with a high correlation coefficient (Eq. 4.1, $R^2 = 0.9866$).

$$Y_{oil}(\%) = 26.9 + 4.2X_1 + 1.6X_2 - 1.4X_3 + 1.4X_1X_2 - 0.8X_1X_3 - 0.9X_2X_3 - 2X_1^2 + 1.9X_2^2 - 4.5X_3^2 \quad \textbf{(4.1)}$$

where X_1, X_2 and X_3 are the coded values for incubation temperature, enzyme amount, and buffer solution pH , respectively.

Table 4.10: Regression coefficients of the second order polynomials for response

Regression Coefficients	Effect	SD	*t* Ratio	*P*	Regression coefficient values
Mean/Intercept (β_0)	26.92958	0.352439	76.40925	0.000000	26.92958
(1)Incubation temperature (β_1)	8.42000	0.520920	16.16371	0.000001	4.21000
Incubation temperature (β_{11})	-3.95352	1.006387	-3.92843	0.005687	-1.97676
(2)Enzyme amount (β_2)	3.22000	0.520920	6.18137	0.000453	1.61000
Enzyme amount (β_{22})	3.84648	1.006387	3.82207	0.006525	1.92324
(3)Buffer solution pH (β_3)	-2.78000	0.520920	-5.33671	0.001080	-1.39000
Buffer solution pH (β_{33})	-8.95352	1.006387	-8.89670	0.000046	-4.47676
1L by 2L(β_{12})	2.85000	0.582406	4.89349	0.001766	1.42500
1L by 3L(β_{13})	-1.70000	0.582406	-2.91892	0.022374	-0.85000
2L by 3L(β_{23})	-1.75000	0.582406	-3.00477	0.019808	-0.87500
Correlation coefficient (R^2) = 0.98659					

Out of three parameters that were examined only buffer solution pH had a negative effect while incubation temperature and enzyme amount had a positive effect on the oil amount (p<0.01). Incubation temperature was the most significant factor (β_1=8.42), followed by enzyme amount (β_2=3.22) and pH (β_3= -2.78). However, the coefficients of interactive terms (X_1X_3 and X_2X_3) had negative signs whereas (X_1X_2) had a positive sign. A positive sign represents a synergistic effect while a negative sign represents an antagonistic effect. On the other hand, X_1X_2 had a statistically significant effect (p<0.01) while X_1X_3 and X_2X_3 had less statistically significant effects (p<0.05) on the oil amount. Other significant effects (p<0.05) are provided by quadratic of incubation temperature, enzyme amount and pH. Thus, incubation temperature, enzyme amount and pH of the buffer solution influence the oil yield during aqueous oil extraction assisted by Celluclast 1.5L from safflower seeds.

The effects of the studied variables on the oil amount are illustrated on the response surface plots and contour plots. On the response surface plots, the levels of variables are shown on three axes (3-D) while the independent variables are shown on the x and y axes (2-D) in contour plots. The oil amount is displayed by the color levels of the related areas on the contour plots. In each graph, the effect of the third variable not displayed was kept constant at its critical level.

The effect of incubation temperature and enzyme amount on the amount extracted is demonstrated on the contour plot and response surface plot in Figures 4.10a-b.

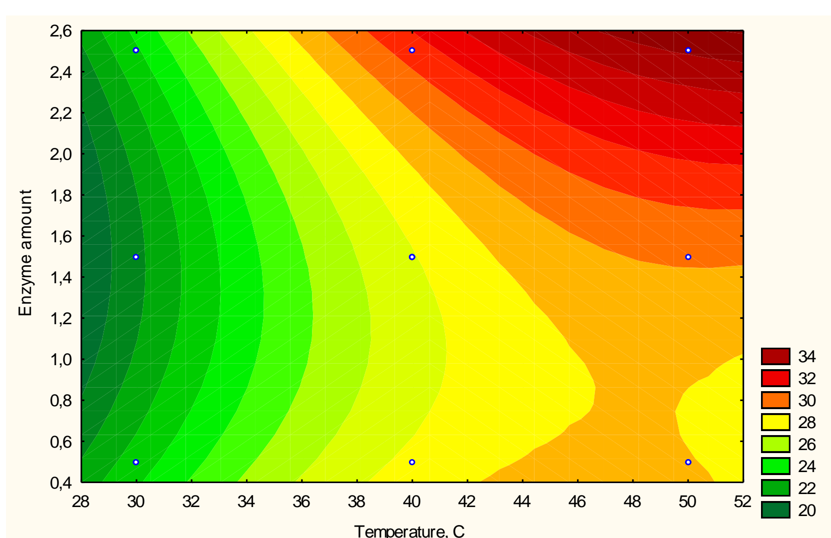

Figure 4.10a: Contour plot for oil amount (% w/w) as a function of incubation temperature (°C) and enzyme amount (mL enzyme/g substrate).

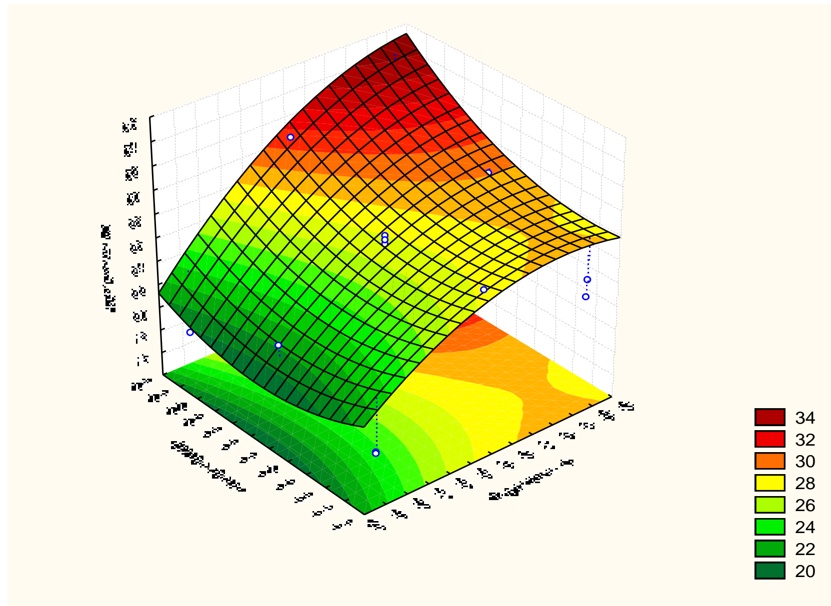

Figure 4.10b: Response surface plot for oil amount (% w/w) as a function of incubation temperature (°C) and enzyme amount (mL enzyme/g substrate).

According to this contour plot, the amount of oil extracted increases with an increase in the enzyme amount. The highest oil amount is obtained between the temperatures 38-52 °C when the enzyme amount exceeds 2.4 mL. It can also be observed that temperature is more critical than enzyme amount. At the response surface plot, it is also observed that temperature is more critical in oil the amount obtained than enzyme amount. Enzyme amount has almost no effect until a temperature of 32 °C.

The effect of the incubation temperature and buffer solution pH on the oil amount is demonstrated on the contour plot and response surface plot in Figure 4.11a-b.

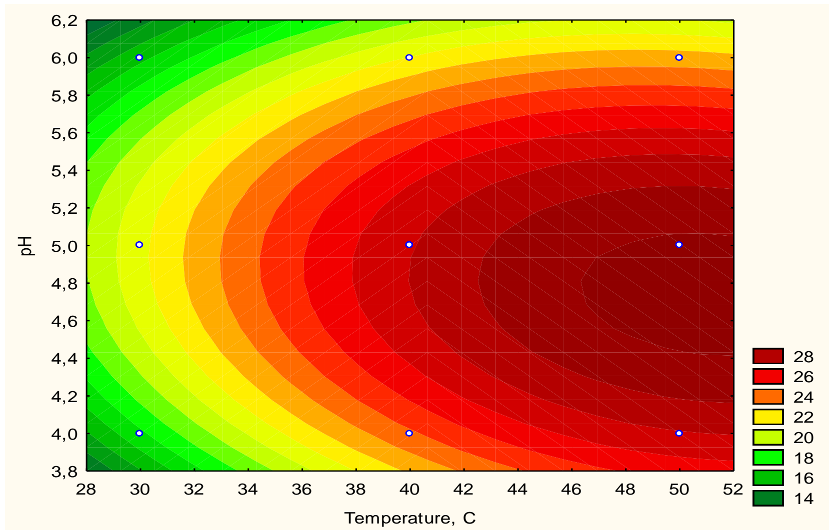

Figure 4.11a: Contour plot for oil amount (% w/w) as a function of incubation temperature (°C) and buffer solution pH.

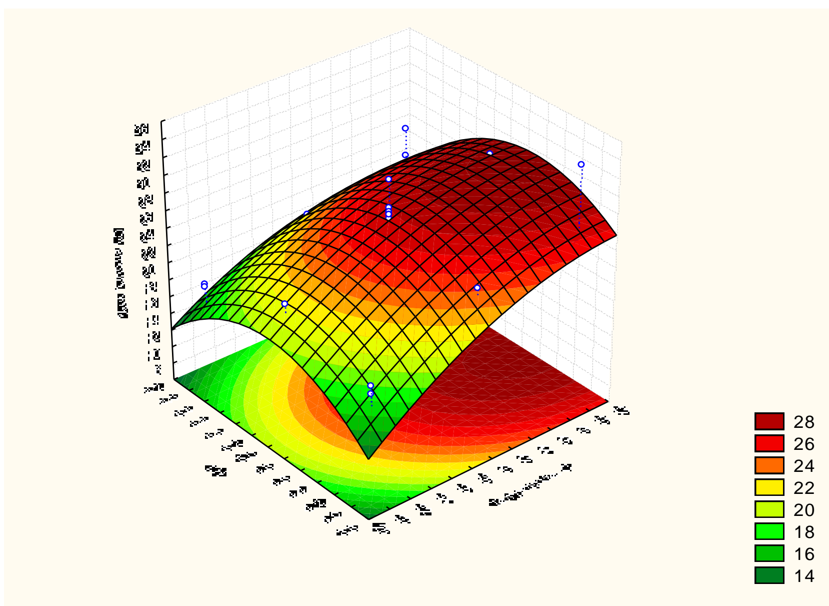

Figure 4.11b: Response surface plot for oil amount (% w/w) as a function of incubation temperature (^{0}C) and buffer solution pH.

According to this contour plot, the oil amount increases with higher temperatures of incubation, and reaches a maximum value after 50 °C. Also, maximum oil amount is estimated with a pH between 4.3-5.2 and a temperature between 47-55 °C. Regarding the response surface plot, it can clearly be observed that the oil amount is maximum between pH values of 4.3 and 5.2 and incubation temperature is more critical than buffer solution pH.

The effect of enzyme amount and buffer solution pH on the oil amount is demonstrated on the contour plot and response surface plot in Figure 4.12a-b.

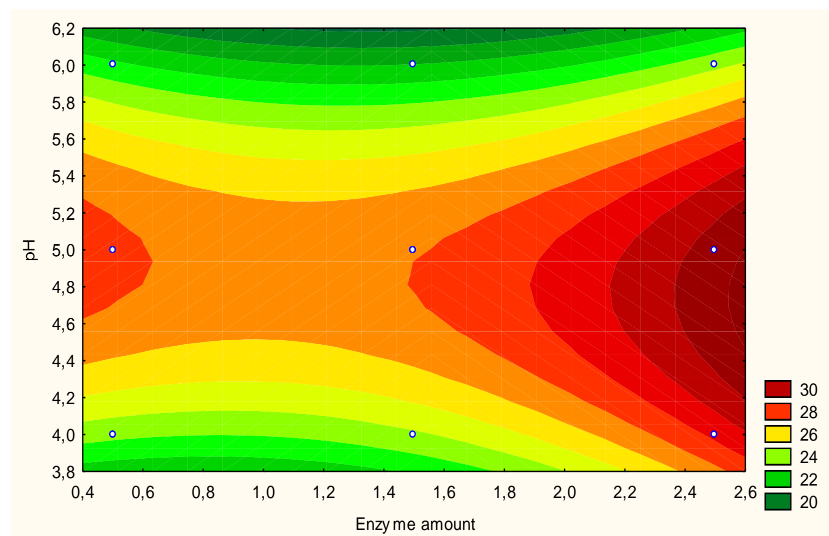

Figure 4.12a: Contour plot for oil amount (% w/w) as a function of enzyme amount (mL enzyme/g substrate) and buffer solution pH.

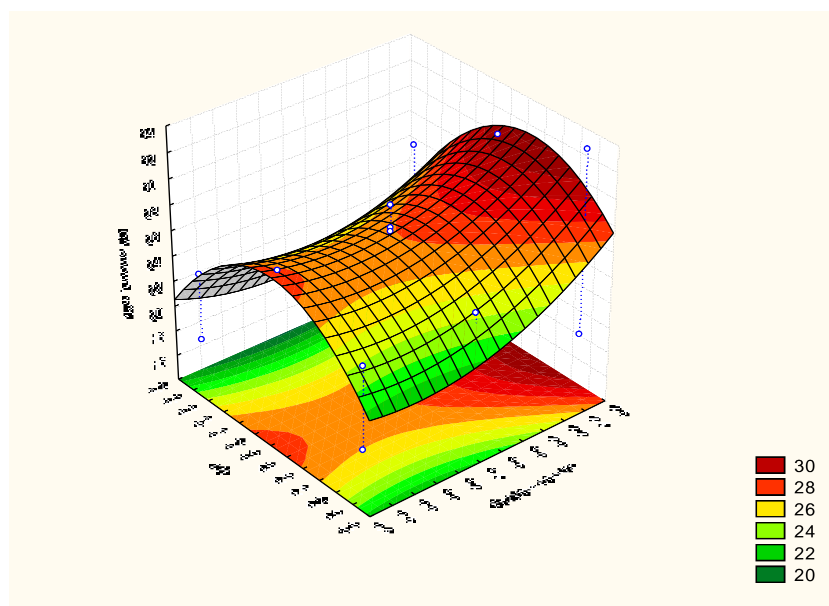

Figure 4.12b: Response surface plot for oil amount (% w/w) as a function of enzyme amount (mL enzyme/g substrate) and buffer solution pH.

In keeping with the contour plot, maximum oil amount is estimated with a pH between 4.3-5.2 and enzyme amount between 2.4-2.6. It can also be observed that enzyme amount is more critical for oil amount. At the response surface plot, it can again be seen that enzyme amount is more critical.

Table 4.11 displays the computed observed minimal, critical and observed maximal values for the three independent variables according to RSM. The optimum conditions were as follows: incubation temperature , 48.3 °C; enzyme amount, 0.74 mL⁄3 g substrate (0.6321 EGU⁄3 g substrate), and pH value of 4.84. At this critical point, 28.2% oil amount (67.5% oil yield) was obtained. Confirmation of the model led to 27.1% oil amount (65% oil yield) after a treatment of 3 g of ground safflower in 20 mL buffer solution with Celluclast 1.5L for 6 h.

Table 4.11: Observed minimal, critical and observed maximal values

Independent variables	Observed minima	Critical value	Observed maxima
Incubation temperature (°C)	30	48.25	50
Enzyme amount (mL enzyme⁄g substrate)	0.50	0.74	2.50
Buffer solution pH	4.00	4.84	6.00

Figure 4.13 plots the observed experimental values of oil obtained from aqueous enzymatic extraction (% w⁄w) vs. the corresponding predicted values computed according to the model in Eq. 4.2 (Table 4.10). The figure indicates that the values of the response predicted from the empirical model are in a good agreement with the observed values in the range of the operating variables with a high correlation coefficient, R^2 = 0.9866. This statistics show that the model, as fitted, explains 98.66% of the variability in the oil amount. Also, the sum of residuals was found to be 0.4. These results indicate the conformity of the computed statistical model.

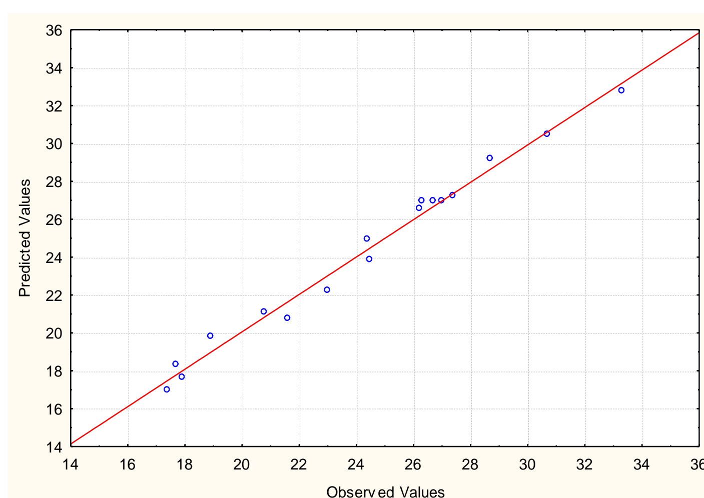

Figure 4.13: Correlation of experimental and predicted oil amount (% w⁄w).

4.4 Characterization of Extracted Oils

The physicochemical properties of safflower oil obtained by the Soxhlet extraction and aqueous enzymatic extraction were compared in respect to acid value (AV), saponification value (SV), refractive index (RI), unsaponified matter (UM), and free fatty acid composition (FFA). As expected, no significant variations were observed for saponification value, refractive index, and unsaponifiable matter of the oils extracted by different means. The physical and chemical parameters of the extracted oils are given in (Table 4.12 and 4.13). The results were found to be in agreement with the findings of Latif *et al.*[34] and Jovanovic *et al.* [38]. Womeni *et al.* [27] pointed out that variations in properties may stem from the differences in the variety of plants, growth climate, ripening stage, the harvesting period of the seeds and extraction conditions.

Table 4.12: Some physicochemical properties of safflower seed oils [37]

Safflower variety	Soxhlet extraction				Aqueous enzymatic oil extraction			
	AV (mg KOH/g oil)	SV (mg KOH/g oil)	RI (25 ℃)	UM (%)	AV	SV	RI	UM
Dincer	2.3	180.5	1.4750	0.6	2.2	177.8	1.4752	0.5
Remzibey	3.6	174.5	1.4762	0.4	-	-	-	-
Yenice*	2.9	189.2	1.4763	0.9	-	-	-	-
614.4*	1.4	193.3	1.4763	-	-	-	-	-
Literature*	0.4-10	188-194	1.469-1.477	0.3-1.3	-	-	-	-

As it can be seen from Table 4.13, no significant difference was observed in the fatty acid composition of the oils extracted by two methods. However, a slightly lower content of free fatty acid was observed in the enzyme extracted oil as against solvent extracted oil. Latif *et al.* [34] expressed that this might be due to accelerated temperature treatment during the solvent extraction.

Table 4.13: FFA composition (grams per 100 g of fatty acids) of extracted oils

Safflower variety	Soxhlet extraction				Aqueous enzymatic oil extraction			
	Palmitic acid (16:0)	Stearic acid (18:0)	Oleic acid (18:1)	Linoleic acid (18:2)	Palmitic acid (16:0)	Stearic acid (18:0)	Oleic acid (18:1)	Linoleic acid (18:2)
Dincer	6.8	2.3	13.9	77.0	6.2	2.1	12.7	78.9

5. CONCLUSIONS AND RECOMMENDATIONS

The objective of this study was to produce good quality oil in an economically viable and environmentally-friendly way. For this purpose, aqueous enzymatic oil extraction of safflower was conducted and the optimization of operational variables was studied via response surface methodology. In this study, commercial enzymes Alcalase 2.5L and Celluclast 1.5L were used to observe their effects on the extracted oil amount but the optimization was performed with Celluclast 1.5L. This selection was made because the effects of the operational variables on the oil amount were more drastic. The physicochemical properties of the oils extracted by Soxhlet extraction and AEE were analysed and compared in terms of their acid value, saponification value, unsaponified matter %, free fatty acid composition and refractory index. It was found that the enzyme treatment did not have any determining effect on these properties.

In this research, a three-factor, three-level cubic central composite design requiring a total of 17 design points with 3 centre points (0,0,0) was adopted for the optimization of aqueous enzymatic oil extraction. The combined effect of enzyme amount (mL enzyme⁄g substrate), buffer solution pH and incubation temperature (°C) (independent variables) on oil amount (response, dependent varieble) was evaluated by this method. The obtained experimental data were computed using Statistica 7.0 software to fit the second-order polynomial model predicted for optimization.

As a result of the optimization, the maximum oil amount and yield were 33.3 (% w⁄w) and 79.7 (% w⁄w), respectively. Incubation temperature was the most significant factor on the oil amount extracted followed by enzyme amount and buffer solution pH. The critical values of the independent variables were as follows: incubation temperature, 48.3 °C; enzyme amount, 0.74 mL enzyme⁄3 g substrate (0.6321 EGU); buffer solution pH, 4.84. At this critical point, 28.2 (% w⁄w) oil amount was observed. The predicted critical values were experimentally verified and an oil amount of 27.1±0.9 (% w⁄w) was achieved. The experimental data for oil

extraction yield obtained with Celluclast 1.5L correlated very well with process parameters, resulting in a model with high correlation coefficient for the oil extraction amount ($R^2 = 0.9866$).

In terms of further research, the investigation of the effects of different enzymes (maybe enzyme preparations such as Viscozyme or other wall degrading enzymes and phospholipases) and pretreatment steps (dehulling, flaking and extruding, hydrothermal pretreatment) that may be applied prior to the enzymatic treatment to increase the oil amount may be pertinent. The de-emulsification and product recovery steps essentially determine the viability of aqueous and enzymatic extraction. If emulsions have formed during extraction, they must be broken down to release the oil. Emulsions can be broken down by several methods such as separating by extraction with *n*-hexane and freezing and thawing. However, new de-emulsification methods can be developed for oil recovery.

Furthermore, the high percentage of oil makes safflower seeds a potential exploitable source of lipids in the industry. Enzyme-aided aqueous extraction processes can be advantageous for extracting oil from oil-bearing material, especially when environmental and safety issues are considered. Good recovery in this environmentally friendly process shows that it is possible to avoid solvents which are harmful to the environment. The oil quality obtained is also found to be similar to solvent extraction. However, pilot plant–scale investigation should be undertaken to evaluate the commercial potential of the process with respect to the extraction and separation steps. In addition, research is needed to simplify the oil recovery steps and make the process commercially more attractive. At the moment, the cost of the enzyme and lower oil amounts are major factors that may prevent the adoption of this technology. When the cost or availability of commercial enzymes is a major concern, crude enzyme preparations containing several enzymes from selected microorganisms can be used. However, increasing environmental concerns coupled with the development of more efficient downstream processing technology for enzymes are likely to make this a viable technology for oil extraction in the future.

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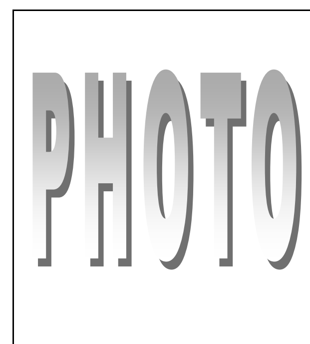

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