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Pretreatment of cottonseed flakes with proteases and an amylase for higher oil yields

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RESUMEN

Pretratamiento de hojuelas de semilla de algodón con proteasas y una amilasa para el incremento del rendimiento de aceite.

En este trabajo se estudió el efecto del pretratamiento con enzimas sobre la extractabilidad del aceite en hojuelas de semilla de algodón. Las enzimas que se investigaron fueron proteasa bacteriana (Bp), papaína (Pa), savinasa (S), temamil (T), pectinasa (Pe) y celulasa (C). Las variables estudiadas durante los experimentos de hidrólisis enzimática fueron: concentración de la enzima, ratio humedad:cantidad de hojuelas y tiempo de hidrólisis. Estos experimentos se realizaron primeramente con una sola enzima y posteriormente con mezclas de enzima formuladas de acuerdo a los resultados obtenidos en los estudios con un solo componente enzimático. Los resultados se evaluaron en función de del incremento relativo de la extractabilidad del aceite, así como de las características del mismo frente a un control realizado con hojuelas sin tratamiento. El pretratamiento con mezclas de enzima resultó en un incremento relativo de la extractabilidad del aceite frente al control mayor del que se registró en los tratamientos con una sola enzima. El análisis estadístico mostró diferencias en extractabilidad significativas (al nivel del 5%) entre el control y las distintas mezclas de enzima. El incremento relativo de extractabilidad debido al pretratamiento con mezclas de enzima siguieron el orden S: Pe: Bp > S: Pe > S: C: Pe > S: Bp > S: T > S: C > S: Pa con valores de 44.9%, 38.9%, 37.1%, 34.9%, 30.1%, 28.9%, respectivamente. Las características de los aceites obtenidos aplicando pretratamiento enzimático fueron generalmente similares al control en cuanto a composición de ácidos grasos, acidez, índice de yodo e índice de peróxidos.

PALABRAS-CLAVE: Aceite de algodón – Celulasas – Extractabilidad – Pectinasas – Proteasas – Tratamiento enzimático.

SUMMARY

Pretreatment of cottonseed flakes with proteases and an amylase for higher oil yields.

The effect of enzymatic pretreatment of cottonseed flakes on oil extractability was studied. The enzymes investigated included bacterial protease (Bp), papain (Pa), savinase (S), termamyl (T), pectinase (Pe) and cellulase (C). The variables studied during the enzymatic hydrolysis experiments were: enzyme concentration, moisture: cottonseed flakes ratio, and time of hydrolysis. Enzymatic hydrolysis experiments were first carried out with a single enzyme, then with enzyme mixtures formulated according to the results of single enzyme treatments. Results were evaluated based on the relative increase in oil extractability, and some oil characteristics in comparison with untreated cottonseed flakes (control). Pretreatment with enzyme mixtures resulted in a relative increase in oil extractability that was higher than single enzyme pretreatment and the control. Statistical analysis showed a significant difference (at 5% level) between the control and all enzymatically treated oils as well as among different enzymatically treated oils. The relative increase in oil extractability due to pretreatment with enzyme mixtures were in the following order: S: Pe: Bp > S: P > S: C: Pe > S: Bp > S: T > S: C > S: Pa with values 44.9%, 38.9%, 37.1%, 34.9%, 30.1%, 28.9%, respectively. Enzymatic pretreatment of cottonseed flakes resulted in oils with fatty acid composition, acid value, iodine value and peroxide values that were generally comparable to the control.

KEY-WORDS: Amylase – Cellulase – Cottonseed oil – Enzymatic treatment – Extractability – Pectinase – Proteases.

1. INTRODUCTION

With the growing population, the demand for edible oils is constantly increasing especially in developing countries. Along with the increase in demand, there is worldwide interest in healthy foods as well as an awareness of the impact of pollution on human health. This leads to the search for new technologies that are ecofriendly and that would result in healthy food products.

The oilseed industry all over the world is mostly based on the solvent extraction of oil, and generally hexane is the most widely employed solvent. The use of hexane results in the emission of volatile organic compounds (VOCs) that lead to many health hazards (e.g. Carcinogenesis), and cause damage to crops and the environment. The meal resulting from the removal of oil from the oilseed is a rich source of protein. This meal as well as the oil will contain residual hexane that is very difficult to get rid of. Oilseeds need to undergo pretreatments before mechanical expelling or solvent extraction of the oil. The conventional pretreatments of oilseeds may include: dehulling, size reduction and flaking in addition to thermal / hydrothermal treatment, cooking and steaming. The heat treatments involved causes damage to the oil and protein qualities.

Solvent extraction can be replaced by mechanical expelling of the oil, aqueous extraction or supercritical fluid extraction. These processes yield oil and protein of higher oil qualities, because of the milder treatments employed. Mechanical expelling and aqueous extraction produce smaller quantities of oil than solvent extraction, because an appreciable amount remains trapped in the cake. Super critical fluid extraction is still too costly to be applied in all oil extraction plants. An alternative to heat treatment prior to oil extraction, involves enzymatic treatment. Enzymatic pretreatment of oilseeds is expected to break cell walls and facilitate the flow of oil. In oleaginous seeds the oil is usually found inside the vegetable cell linked with other macromolecules such as proteins, pectin, carbohydrates (Mcglone et al., 1986; Badr and Sitohy, 1992). A cell wall study envisions a cellulose-hemicellulose structural domain embedded in a secondary domain consisting of pectic substances, while a third domain consists of covalently cross linked proteins (Carpita and Gibeaut, 1993). Since the structural composition of the cell wall is specific to each oil source, the selection of a suitable enzyme system is critical for efficient oil extraction. Despite the fact that a single type of enzyme may achieve a significant oil recovery, in some cases the combination of several enzymes is often required to degrade a wide range of structural composition in the cell matrix (Chen and Diodsay, 2002).

The use of enzymes as an aid in the oil extraction process has been first recommended by (Hitze et al., 1972), for extraction of oil from corn germ. Moreau et al. (2004) also used enzymatic pretreatment to extract higher yields of corn germ oil. Lanzani et al. (1975) carried out an initial series of preliminary tests on enzymatically hydrolyzing rapeseed, peanuts and sunflower seeds with proteases, cellulases, galactouronide-glicane hydrolase under different conditions of time, temperature, and different concentrations. They reported an increase in oil recovery percentage against total oil under all conditions. Sozulski and Sozulski (1993) and Sengupta and Battacharyya (1996), reported that the quantity of oil extracted after the enzymatic pretreatment of mustard seed and canola was higher than that resulting from untreated seeds. Sitohy et al. (1993) and Dominquez et al. (1993) hydrolyzed sunflower kernels with most of the known effective enzymes as well as multiactivity enzyme complexes and found them to differ in the percentage of extracted oil according to the type of enzyme used.

Several authors examined the effect of enzymatic pretreatment of soybeans for increasing oil extractability. Smith et al. (1993) investigated enzymatic pretreatment followed by mechanical expelling of soybeans. The process parameters were optimized by means of surface methodology. They concluded that enzyme pretreatment enhanced both the amount of extractible oil and oil extractability. A second-order response surface model was developed to predict the expelled oil as a function of six process parameters investigated. Shankar et al. (1997), applied enzymatic hydrolysis in conjunction with flaking (dehulling inherent) and steam conditioning. They reported that it was the best pretreatment compared to other pretreatments they examined.

Bhatnagar and Johari (1987), enzymatically treated cottonseed with several microbial enzymes: proteases, cellulase, and hemicellulase prior to oil extraction. Protease from H. lumuginosa L. resulted in the highest oil extractability. Taha et al. (2002) carried out enzymatic pretreatment of cottonseed flakes with cellulose, hemicellulase, and pectinase. They reported that enzymes, when used individually, resulted in different levels of increasing oil extractability, compared to untreated cottonseed flakes. The highest increase in percent oil extracted was achieved with enzyme mixtures.

Sharma et al. (2002) and Wang et al. (2004) reported positive results for the enzymatic treatment of peanuts preceding oil extraction. Rice bran has been reported to give higher oil yields when stabilized rice bran was treated with enzymes before oil extraction (Sharma et al 2001; Hanmoungjai et al. 2002). Coconut and Shea kernel were also subjected to enzymatic pretreatment which proved to positively increase oil extractability (McGlone et al., 1986; Tano-Debrah and Ohta, 1995; CheMan et al., 1996).

In order to achieve an environmentally friendly process, the enzymatic pretreatment of oilseeds should be followed by any process to extract the oil other than solvent extraction especially when using hexane. Extracting the oil mechanically or by Centrifugation has been suggested by (Smith et al., 1993; Hanmoungjaj et al., 2002).

It became clear that the enzymatic pretreatment of oilseeds followed by hydraulic pressing would result in higher oil yields, improved oil quality, and a cleaner technology. In a previous paper (Taha et al., 2002), investigated the effect of the pretreatment of cottonseed flakes with the enzymes cellulase, hemicellulase, and pectinase. Their results revealed that the relative increase in oil extractability as a result of enzymatic pretreatment with enzymes and their mixtures were in the following order: Pectinase-cellulase > pectinase > pectinase – cellulase - hemicellulase > cellulase > hemicellulase.

The present investigation is a continuation of the work on cottonseed flakes, where the effect of several proteases (Bacterial protease, papain and Savinase) as well as ·-amylase (Termamyl) on oil yield and quality was investigated. The enzymatic reaction was carried out under different enzyme concentrations (1, 2, and 3 percent), at different moistures: cottonseed flakes ratio (1: 5.5, 1: 7, 1: 10.5 w: w), and for 3 and 6 h. In order to determine the effect of the different enzymes on the parameters investigated, single enzymes and the mixtures formulated with them were used. Enzymatic treatment was followed by solvent extraction of the oil for convenience (solvent extraction is more convenient than hydraulic

pressing). In a coming study, and owing to the optimum results achieved in this work, the optimum enzymatic conditions will be applied on cottonseed flakes followed by hydraulic pressing to reach the original goal of a clean technology.

2. MATERIALS AND METHODS

Cottonseed flakes (*Gossypium barbadence)* were supplied by El- Minya Ginning Company, El-Minya, Egypt.

Enzymes Papain and Bacterial protease are products of SIGMA, USA. Enzymes Savinase and Termamyl are products of NOVOZYME, Denmark. (offered kindly as free samples).

Standard methyl esters were products of SIGMA, USA.

2.1. Enzymatic treatment of cottonseed flakes.

A calculated amount of water was added to the flakes to reach the desired Moisture content (M): Cottonseed flakes(CSF) ratio (w/w ratio), mixed well, the enzyme was then added, the mixture was continuously agitated on a magnetic stirrer, while the pH was being adjusted, continuously for 15-20 min to ensure the stability of pH. The flask containing the reaction mixture was placed in a thermostatic water bath adjusted to the optimum temperature for each enzyme. Shaking continued for a predetermined time. At the end of the experiment the temperature was raised to 105 °C for 30 min and the pH dropped to 4.0 to stop the activity of the enzyme. The hydrolyzed flakes were then filtered through (Whatman no.1) filter paper, left in the open air for about 3h and then placed in a draft air oven at 60 °C for 24h. The dried flakes were then ground to pass 60 mesh screens and subjected to oil extraction using n-hexane in a soxhlet apparatus. The extraction was carried out for 12h, dried at 60 °C; the meal was reground, and re-extracted with a fresh quantity of n-hexane for an additional twelve hours. The two hexane extracts were combined, dried over anhydrous sodium sulphate, filtered and evaporated to near dryness in a rotary evaporator, then dried in a vacuum oven at 60°C overnight, till constant weight, and percent extracted oil was calculated. Variables investigated in this study include: Enzyme concentration 1, 2, and 3 percent were calculated as percent of sample weight, M: CSF ratio w/w, 5.5:1, 7:1, 10.5:1, and duration of hydrolysis 3 and 6 h. Other conditions such as temperature and pH used were those recommended by the manufacturers. For papain 25 °C and pH 6.2; bacterial protease 37 °C and pH 7.5; savinase 55 °- 60 °C and pH 8-11; Termamyl 85°-115°Cand pH 5.6-6.6. A control representing untreated CSF that was directly subjected to oil extraction with n-hexane was also carried out.

This experiment was repeated four times for each enzyme or enzyme mixture under the investigated conditions.

2.2. Formulation of enzyme mixtures

Enzyme mixtures were formulated using bacterial protease (Bp), papain (Pa), savinase (S), termamyl (T), cellulase (C), and pectinase (Pe).

Formulated enzyme mixtures are represented in Table 1.

2.3. Fatty acid composition

The component fatty acids of the oil samples extracted from enzymatically treated cottonseed flakes, together with a control sample resulting from untreated cottonseed flakes were converted to their methyl esters by esterification according to (Christie 1973). The reaction was monitored with the help of TLC to ensure complete conversion to methyl esters. The mixed methyl esters of each sample were subjected to gas liquid chromatographic (GLC) analysis. A Hewlett Packard (HP) Model 6890 Gas Chromatograph was employed for the analysis under the following conditions: INNO wax capillary column (polyethylene glycol), 30.0 m $\times$ 530 µm, film thickness 1.0 µm; column was operated isothermally at 280 °C; injection temperature 280 °C; split ratio 8:1; split flow 120 ml; gas saver 20 ml/min; and carrier gas N_2, with flow rate 15 ml/min; FID detector temperature 280 °C; hydrogen flow rate 30 ml/min; and air flow rate 300 ml/min. Peak areas were determined by electronic integrator and percentage composition of fatty acids automatically calculated. A standard mixture of fatty acids methyl esters (methyl myristate, methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, and methyl linolenate) was also chromatographed under the same operating conditions. The entity of the peaks was achieved through comparison of the retention times with those of standards.

2.4. Oil analysis

Moisture, acid value, iodine value, and peroxide value were determined according to standard methods of analysis of AOCS (1998).

Table 1
Formulation of enzyme mixtures.

Treatment	Enzyme mixture	pH	Temperature
1	S: BP (1:1)	8	40
2	S: Pa (1:1)	7	30
3	S: T (1:1)	7	70
4	S: C (1:1)	6.5	45
5	S: Pe (1:1)	6	35
6	S: Pe: BP (0.66:0.66:0.66)	6.5	50
7	S: Pe: C 0.66: 0.66:0.66)	6.5	50

* These mixtures were carried out usin 2% enzyme concentration, M: CSFratio 7: 1, for 6 hr

Experiments were carried out in four replicates, and analyses of all samples in duplicate.

2.5. Statistical analysis

The experiment followed completely randomized design (CRD). The obtained data were subjected to analysis of variance (ANOVA) according to (Snedcor and Cochran 1980). Duncan's Multiple Range test was used to compare between means of treatments according to (Walter and Duncan 1969) at probability 5 percent.

Correlation studies were done on an HP home computer, using excel program.

3. RESULTS AND DISCUSSION

This work is the continuation of a previous paper (Taha et al., 2002) where the effect of the enzymes C, HC, and Pe on the oil extractability from CSF was studied. Results indicated that enzymes C and Pe exhibited a significant effect on the oil extractability from CSF, while HC was less efficient. Furthermore, a mixture of C and Pe in a 1:1 ratio (w/w) resulted in higher oil extraction than the two single enzymes. In the present work, since the lipids are sometimes bound in the form of lipoproteins and liposaccharide complexes (Shankar et al.1997), it was worth while to investigate the effect of the proteases (Bp, Pa, and S) and a α-amylase (T) on the oil extractability of CSF. Mixtures from the enzymes studied in our previous paper(C, HC, Pe) together with the enzymes under investigation (Bp, Pa, S, T) were formulated and their effect on the oil extractability of CSF was studied.

3.1. Effect of pretreatment of cottonseed flakes with bacterial protease on oil extraction

Table 2. Shows the results of enzymatically hydrolyzing CSF with Bp at (1, 2, and 3 percent enzyme concentration), M: CSF ratio (5.5: 1, 7: 1, and 10.5: 1 w/w), for 3 and 6h. All treatments resulted in an increase in the extracted oil from CSF compared to the control (oil from untreated CSF). There was a significant difference at (5% level) among all treatments and the control. Significant and non significant difference between treatments can also be detected from Table 2. Only the treatment of CSF with 3% Bp, at M: CSF ratio 7:1, for 6h showed a highly significant difference with all other treatments. Oil extracted from control sample was 20.5%, while enzymatic treatment with Bp under the different conditions of the investigation resulted in extracted oil between 21.5-25.9%. The highest relative increase in oil extractability 26.1% was achieved at 3 % enzyme concentration, M: CSF ratio 7:1 and duration of 6h. Treatments with Bp at 2% and 3% concentration, at 7:1 M: CSF ratio, for 3 and 6h resulted in a percent increase in oil extractability between 21.6 and 22.6%. Treatment with 1% enzyme concentration, 10:1 and 5.5: 1 M: CSF ratio, for 3h and 6h also resulted in a

Table 2
Effect of bacterial protease pretreatment of cottonseed flakes on oil extractability.

Enzyme conc.(%)	Time (hrs)	M : CSF ratio	Extracted oil (%)	Increase in oil extractability (%)
1	3	5.5:1	21.49 ± 0.37g	4.77
1	3	7: 1	23.13 ± 0.69 f	12.73
1	3	10.5: 1	24.88 ± 0.64 bc	21.29
2	3	5.5:1	22.92 ± 0.60 f	11.71
2	3	7: 1	24.96 ± 0.38 bc	21.67
2	3	10.5: 1	23.91 ± 0.83 de	13.26
3	3	5.5:1	23.24 ± 0.51 ef	13.26
3	3	7: 1	25.16 ± 0.49 b	22.64
3	3	10.5: 1	24.39 ± 0.76 cd	18.85
1	6	5.5:1	25.02 ± 0.47 bc	21.92
1	6	7: 1	22.83 ± 0.68 f	11.28
1	6	10.5: 1	22.03 ± 0.68 g	7.38
2	6	5.5:1	23.22 ± 0.48 ef	13.16
2	6	7: 1	25.10.5 ± 0.55 bc	22.33
2	6	10.5: 1	24.39 ± 0.35 c d	18.85
3	6	5.5:1	21.99 ± 0.47 g	7.18
3	6	7: 1	25.88 ± 0.56 a	26.11
3	6	10.5: 1	23.35 ± 0.28 ef	13.8
Control (untreated flakes)			20.52 ± 0.58 h	

M = moisture, CSF = cottonseed flakes. Means with different letters within each column are significant, means followed by the same alphabetical letters are not significantly different at 5% level and means without letters are not significant. SD : Calculated from values of four replicates.

percent increase in oil extractability of 21 and 22%, respectively. Treatment of CSF with Bp under the other investigated conditions gave lower relative increase in oil extractability.

3.2. Effect of pretreatment of cottonseed flakes with papain on oil extraction

Results in Table 3. reveal that the pretreatment of CSF with Pa enzyme at different enzyme concentrations, M: CSF ratio, and different times, produces that highest quantity of extracted oil ca.25% was achieved at 3% Pa concentration, 5.5:1 M: CSF ratio, and 3 h of hydrolysis. All treatments showed a significant difference at (5% level) compared to the control, except for treatment with Pa at 1% enzyme concentration, 5.5:1 M: CSF ratio, for 3 h which showed no significant difference with control. Significant and non significant differences between treatments can be seen clearly in Table 3. Treatment with Pa under all the investigated conditions resulted in oil extraction from 22 to 25%. The highest relative increase in oil extractability ca. 23 % was attained when enzymatic hydrolysis was carried out at 3% enzyme concentration, 5.5: 1 M: CSF ratio and hydrolysis continued for 3 h. Other values of relative increase in oil extractability ranged from 7.25-22.7%.

3.3. Effect of pretreatment of cottonseed flakes with savinase on oil extraction

Table 4. Indicates that pretreatment of CSF with S achieved the highest yield of extracted oil 26.2% at 1% enzyme concentration, 7: 1 M: CSF ratio, and time of 3 h. There was a significant difference at (5% level) between the oil extracted under the previous conditions and the control. There was also a significant difference between the oil extracted at 3% enzyme concentration, 5.5: 1 M: CSF ratio, for 6 h and the control oil. As seen from the table, there was no significant difference between other treatments and the control, as well as no significant difference among most of the treatments. The highest relative increase in oil extractability reached ca. 27.7%, other values of relative increase in oil extractability for other treatments ranged from 8.0-23.9%.

3.4. Effect of pretreatment of cottonseed flakes with termamyl on oil extraction

Depicts the way in which the pretreatment of CSF with enzyme T prior to oil extraction resulted in the highest relative increase in oil extractability of 25%. This was achieved under the following conditions: 2 and 3% enzyme concentration, 5.5: 1 M: CSF ratio with a treatment duration of 6 h. 3% enzyme concentration, 7: 1 M: CSF ratio, for 3 h also resulted in 25% oil extractability. Naturally there was no significant difference at (5% level) among these three treatments, but there was a significant difference among them and the other treatments compared to the control. All treatments exhibited a significant difference compared to the control. The relative increase in oil extractability resulting from the different treatments ranged between 7.78-23.94%.

Table 3
Effect of papain pretreatment of cottonseed flakes on oil extractability.

Enzyme conc.(%)	Time (hrs)	M : CSF ratio	Extracted oil (%)	Increase in oil extractability (%)
1	3	5.5:1	22.33 ± 0.34 f	8.83
1	3	7: 1	24.01 ± 0.31 c	17.01
1	3	10: 1	22.01 ± 0.67 e	7.25
2	3	5.5:1	24.58 ± 0.33 bc	19.8
2	3	7: 1	22.42 ± 0.32 e	9.23
2	3	10: 1	23.11 ± 0.45 d	12.61
3	3	5.5:1	25.19 ± 0.43 a	22.74
3	3	7: 1	23.05 ± 0.47 d	12.19
3	3	10: 1	22.37 ± 0.50 e	8.99
1	6	5.5:1	23.13 ± 0.56 d	12.7
1	6	7:1	24.21 ± 0.50 c	17.98
1	6	10:1	23.14 ± 0.60 d	12.76
2	6	5.5:1	23.27 ± 0.04 d	13.4
2	6	7:1	25.07 ± 0.34 ab	22.17
2	6	10:1	23.23 ± 0.54 d	13.23
3	6	5.5:1	24.22 ± 0.40 c	18.03
3	6	7:1	25.04 ± 0.30 ab	22.05
3	6	10:1	23.18 ± 0.53 d	12.98
Control (untreated flakes)			20.52 ± 0.58 f	

M = moisture, CSF = cottonseed flakes. Means with different letters within each column are significant, means followed by the same alphabetical letters are not significantly different at 5% level and means without letters are not significant. SD : Calculated from values of four replicates

Table 4
Effect of Savinase pretreatment of cottonseed flakes on oil extractability.

Enzyme conc.(%)	Time (hrs)	M : CSF ratio	Extracted oil (%)	Increase in oil extractability (%)
1	3	5.5:1	22.18 ± 0.47 def	8.09
1	3	7:1	26.21 ± 0.27 a	27.73
1	3	10:1	23.47 ± 0.53 bcde	14.351
2	3	5.5:1	23.85 ± 0.69 abcde	16.21
2	3	7:1	25.05 ± 0.81 abc	22.09
2	3	10:1	22.39 ± 0.88 cdef	9.11
3	3	5.5:1	22.15 ± 0.55 def	7.93
3	3	7:1	23.25 ± 0.49 bcdef	13.29
3	3	10:1	21.96 ± 0.68 def	7.01
1	6	5.5:1	23.08 ± 0.67 abcdef	12.45
1	6	7:1	22.63 ± 1.37 cdef	10.3
1	6	10:1	21.41 ± 0.64 ef	4.32
2	6	5.5:1	24.45 ± 0.51 abcd	19.13
2	6	7:1	21.73 ± 0.64 def	5.91
2	6	10:1	22.55 ± 0.48 cdef	9.91
3	6	5.5:1	25.43 ± 0.61 ab	23.95
3	6	7:1	22.18 ± 0.84 def	8.08
3	6	10:1	23.29 ± 0.64 bcdef	13.49
Control (untreated flakes)			20.52 ± 0.64 f	

M = moisture, CSF = cottonseed flakes. Means with different letters within each column are significant, means followed by the same alphabetical letters are not significantly different at 5% level and means without letters are not significant. SD : Calculated from values of four replicates.

3.5. Effect of pretreatment of cottonseed flakes with enzyme mixtures on percent increase in oil extractability

From the above results it can be concluded that optimum oil extractability was achieved with the four investigated enzymes in the following order S > Bp > T > Pa.

Enzyme mixtures were formulated with the S enzyme being the common partner in all mixtures since the results of single enzymatic reactions revealed that this enzyme produced the greatest amount of oil extraction. Enzyme mixtures are formulated as shown Table 1. Results of pretreatment of CSF with enzyme mixtures are demonstrated in Figure 1. Mixture S: Pe: Bp resulted in the highest relative increase in extracted oil reaching 44.9 %, as compared to the control. Relative increases in extracted oil from CSF when pretreated with enzyme mixtures were in the following order : S : Pe : Bp > S : Pe > S : C : Pe > S : Bp > S : T > S : C > S : Pa with values of 44.9%, 39.7%, 38.9%, 37.1%, 34.9%, 30.1%, 28.9%, respectively.

From the previous results it is obvious that when using single enzymes for the enzymatic pretreatment of cottonseed flakes, the proteases resulted in higher oil yields. Investigated proteases S, Bp, and Pa gave relative increases in oil extractability over the control oil of 28%, 26%, and 23%, respectively. While single enzymes T, Pe, and C resulted in 24%, 23%, and 10.5% relative increase in oil extractability, respectively, (results for C and Pe are from our previous study Taha et al. 2002). Bahatnagar and Johari (1987), enzymatically extracting cottonseed oil with several proteases from microorganisms as well as cellulase and hemicellulase, reported that the protease from *H.lanuginosa-I* gave the highest oil yield compared to other enzymes and the control. Other investigators reporting proteases to yield higher oil quantities than other enzymes studied include: Hanmoungjiai et al (2002), working with rice bran; Santas and Ferrari (2005), working with soybean; Sharma et al. (2002), working with peants; and Lanzani et al. (1975), working with rapeseed and peanut; and Sitohy et al. (1993) working with sunflower.

In an attempt to explain the fact that proteases can be more effective than carbohydrases and pectinases although the cell wall is made up of

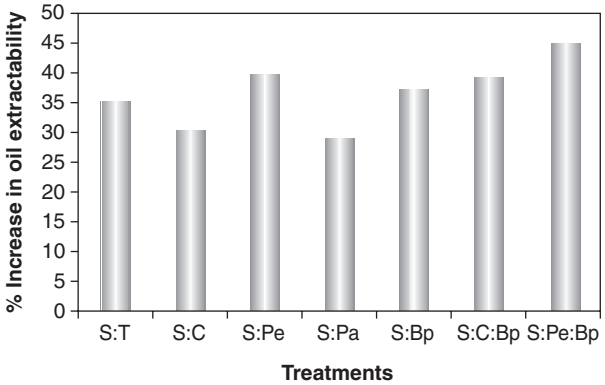

Figure 1
Effect of pretreatment of cottonseed flakes with enzyme mixtures on increase in oil extractability.

cellulose, hemicellulose, lignin and pectin (Snyder and Kwon 1987), other authors report the presence of protein in the cell wall. It is worthy to note that the main feature of oilseed cotyledon cells is the existence of discrete cellular organelles called lipid and protein bodies which contain all the oil and protein in the grain. It is also important to know that oil bodies contain abundant proteins called oleosins which seem to play an important role in stabilizing these bodies. Proteolytic enzymes can also affect the cytoplasmic network which is largely composed of protein in oilseeds, or break up the lipid protein complexes when present (Rosenthal et al.1996).

The increase in relative oil extractability due to the use of mixed enzymes over single enzymes can be depicted from our results where treatment 6 (S: Pe: Bp) resulted in 44% relative increase in oil extractability while S , Pe, Bp when used as single enzymes gave 28%, 23%, 26%, respectively. The work of many authors confirm this finding (Lanzani et al.1975; Che Man et al.1996; Sharma et al. 2001; Hanmoungjiai et al.2001; Taha et al 2002). These results are logical since the oil cell wall is made up of cellulose, hemicellulose, pectin, lignin and protein . Therefore the treatment of the cells of the oilseeds with several of the specific enzymes needed to break up these constituents will free more oil (Rosenthal et al. 1996)

3.6. Results regression

Figures 2 a,b,c and d depict the fact that that the relationship of enzyme concentration versus the increase in oil extractability have a linear correlation, with fair regression coefficient which ranged from 0.92 to 0.99 except in the following cases: figure 2a., savinase enzyme at these conditions of treatment, 3h, 10.5: 1 M: CSF and 6h, 10.5: 1 M: CSF; figure2b., papain enzyme at 3h, 7:1 M: CSF and 3h, 10.5:1 M: CSF; figure 2c., savinase enzyme at 3h, 10.5:1 M: CSF and 6h, 7:1 M: CSF; figure 2d. Termamyl enzyme at 3h, 7:1 M: CSF, 3h, 5.5:1 M: CSF, 6h, 7: 1 M: CSF. These cases are obviously non-linear relationships which were best fitted by using least square method and their perfect regression coefficient is higher than 0.9999.

3.7. Effect of enzymatic pretreatment of cottonseed flakes on the characteristics of the extracted oil

Results in Table 6 reveal the effect of pretreatment of CSF with single enzymes under optimum conditions, as determined by the highest relative increase in extracted oil, on some oil characteristics. Table 6 also indicates the effect of pretreatment with enzyme mixtures (under the conditions given in table 1) on some characteristics of the extracted oil.

3.7.1. Iodine value (IV)

The IV indicates the degree of unsaturation of oils. The IV of the oils resulting from most of the emzymatic treatments (Table 6) showed no

Table 5
Effect of termamyl pretreatment of cottonseed flakes on oil extractability.

Enzyme conc.(%)	Time (hrs)	M : CSF ratio	Extracted oil (%)	Increase in oil extractability (%)
1	3	5.5:1	23.71 ± 0.71 bcd	15.56
1	3	1:7	23.91 ± 0.73 bcd	16.48
1	3	1:10.5	22.318 ± 1.042 fg	8.76
2	3	5.5:1	24.29 ± 0.42 bc	18.39
2	3	1:7	22.39 ± 0.81 fg	9.15
2	3	1:10.5	22.48 ± 0.82 efg	9.55
3	3	5.5:1	22.12 ± 0.54 g	7.78
3	3	1:7	25.36 ± 0.60 a	23.56
3	3	1:10.5	23.45 ± 0.55 cde	14.25
1	6	5.5:1	22.95 ± 0.76 defg	11.84
1	6	1:7	22.45 ± 0.70 fg	9.41
1	6	1:10.5	24.24 ± 0.81 bc	18.14
2	6	5.5:1	25.35 ± 0.98 a	23.52
2	6	1:7	24.52 ± 1.05 ab	19.47
2	6	1:10.5	23.23 ± 0.60 def	13.22
3	6	5.5:1	25.43 ± 0.79 a	23.94
3	6	1:7	23.00 ± 0.78 defg	12.11
3	6	1:10.5	22.43 ± 0.87 fg	9.33
Control (untreated flakes)			20.52 ± 0.58 h	

M = moisture, CSF = cottonseed flakes. Means with different letters within each column are significant, means followed by the same alphabetical letters are not significantly different at 5% level and means without letters are not significant. SD : Calculated from values of four replicates.

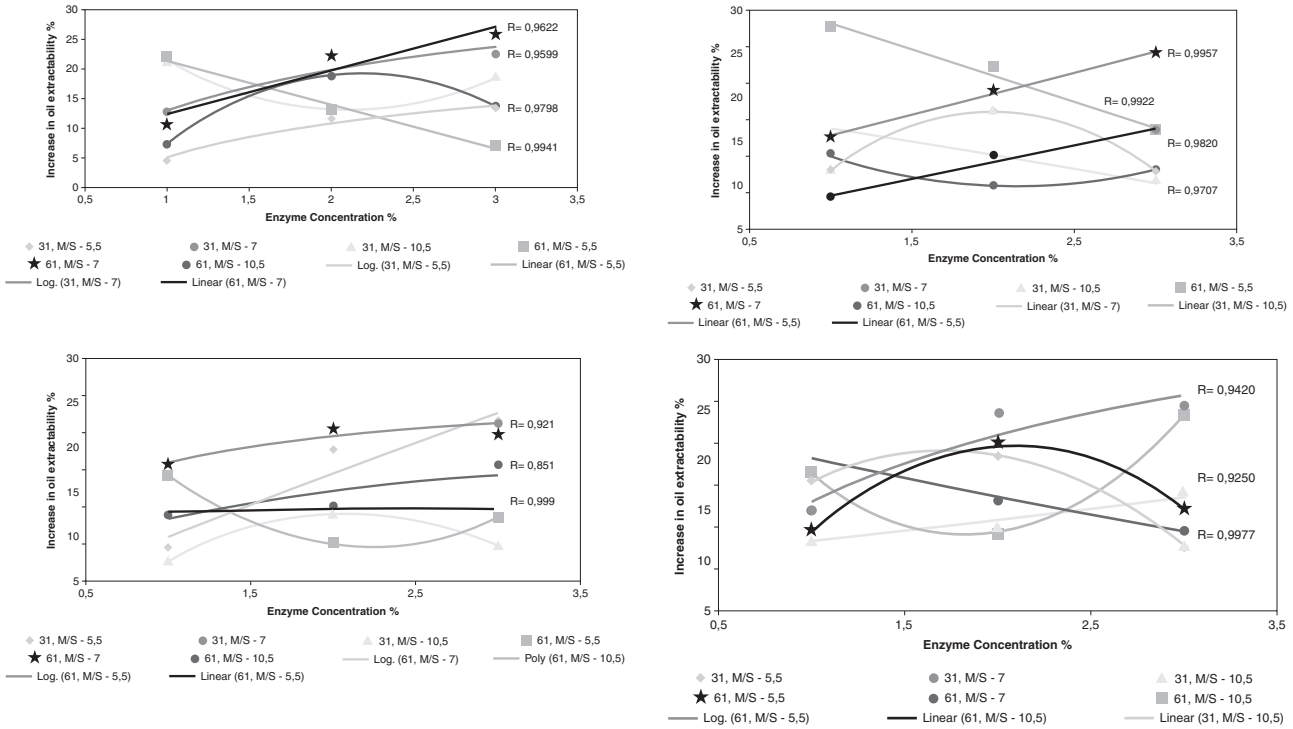

Figura 2
a) Effect of bacterial protease concentration on increase in oil extractability; b) Effect of papain concentration on increase in oil extractability; c) Effect of savinase concentration on increase in oil extractability; d) Effect of Termamyl Enzyme Concentration on Cotton Seed Oil Extractability %.

significant difference (at 5% level) when compared to the control oil (untreated). The oils resulting from the pretreatment with S and Bp exhibited a significant difference compared to the control and other treatments. The IV of the control oil was 98.50, while oils resulting from most of the treatments were less than the control with IV ranging from 95.6 to 98.6. The IV of the oils resulting from pretreatment with enzyme mixtures S: Pe and S: Bp: Pe was slightly higher (99.0) than the control.

3.7.2. Acid value (AV)

The oil hydrolysis (as indicated by the AV) as a result of pretreatment with single enzymes or their mixtures indicate that there was no or very slight hydrolysis (Table 6). There was no significant difference (at 5% level) between treatments and the control nor among treatments. Only the AV of the oil resulting from treatment 5. Showed a significant difference with that of the control oil and oils resulting from other treatments.

Table 6
Effect of Enzymatic Pretreatment of Cottonseed Flakes on Some Characteristics of the Extracted Oil.

Enzyme Treatment	Iodine Value	Acid Value	Peroxide Value
non (control)	98.50 ± 0.1 ab	4.93 ± 0.2abc	0.0h
Bacterial protease	95.62 ± 0.12 d	5.30 ± 0.09a	0.28 ± 0.13 g
Savinase	96.03 ± 0.20cd	5.27 ± 0.49a	1.17 ± 0.12 f
Papain	97.27 ± 0.16bc	4.65 ± 0.10cd	1.27 ± 0.14 ef
Termamyl	97.70 ± 0.14ab	5.18 ± 0.12a	1.63 ± 0.08 cd
Savinase : Bacterial protease (1:1)	98.57 ± 0.14ab	5.13 ± 0.12ab	1.18 ± 0.08 f
Savinase : Papain (1:1)	98.48 ± 0.26ab	4.9 ± 0.13abc	1.88 ± 0.09 b
Savinase : Termamyl (1:1)	98.30 ± 0.11ab	4.80 ± 0.09bd	1.53 ± 0.05 d
Savinase : Cellulase (1:1)	97.62 ± 0.12ab	5.13 ± 0.1ab	1.35 ± 0.05 e
Savinase :Pectinase (1:1)	99.07 ± 0.48 a	4.57 ± 0.12d	1.92 ± 0.08 b
Savinase:Cellulase:Pectinase (0.66 :0.66 : 0.66)	96.90 ± 0.89bcd	4.97 ± 0.12abc	1.70 ± 0.09 c
Savinase:Bacterial protease: Pectinase (0.66 : 0.66 : 0.66)	99.23 ± 0.15 a	5.0 ± 0.22abc	2.10 ± 0.09 a

Means with different letters within each column are significant, means followed by the same alphabetical letters are not significantly different at 5% level and means without letters are not significant. SD : Calculated from values of four replicates.

3.7.3. Peroxide value (PV)

PV indicates the oxidation of the oil. Results in Table 6 show that after pretreatment of CSF with all enzymes and their mixtures the PV increased to different degrees. Statistical data reveal a significant difference between treatments and the control (at 5% level), as the control had zero PV. Significant difference between some of the treatments could be observed from the results. The increase in PV ranged from 0.28 to 2.10 meq / kg oil.

Tano-Debrah and Ohta (1995); Che Man et al. (1996); Hanmoungjiai et al. (2001); Taha et al. (2002); Moreau et al (2004); and others when analyzing oils of different oilseeds arising from the enzymatic hydrolysis of oil cell wall prior to oil extraction reported the oil characteristics including AV, IV, PV to be the same as or better than the conventionally extracted oils.

3.7.4. Fatty acid composition of oils extracted from enzymatically treated cottonseed flakes

Table 7 reveals the fatty acid (FA) composition of the oils extracted from enzymatically treated CSF, together with oil extracted from non treated CSF. The results of GLC analysis of oils show slight differences between the FA composition of enzymatically treated oils and untreated oil. Also the difference in the FA composition of oils from different treatments is slight. The ratio between Saturated: Unsaturated (S: US) fatty acids was close for most of the different enzymatically treated oil samples and the control. The ratio of S: US FA was higher for oils resulting Pa and S: Bp treated CSF, being 1: 2.48 and 1: 2.34, respectively, compared to 1: 2.23 for untreated oil. These results are in agreement with the work of (Taha et al. 2002) where they reported slight changes or no significant difference between enzymatically treated oils and non treated oils.

4. CONCLUSION

It can be said that the enzymatic pretreatment of oilseeds in general, is a safe and efficient tool for increasing the extracted oil yield as well as producing good quality edible oil. Savinase (a protease) either alone or preferably in combination with pectinase, bacterial protease and cellulase, is recommended for the pretreatment of cottonseed flakes under the conditions reported to increase oil yield and preserve oil quality. Although enzymatic pretreatment in this work was followed by solvent extraction of the oil (this was only for convenience) but will be replaced by hydraulic pressing in am up coming study in order to fully achieve the clean technology we are seeking .

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Table 7
Fatty Acid Composition of Cottonseed Oil Extracted from Enzymatically Treated Cottonseed Oil.

Enzyme Treatment	Fatty Acid Composition %							
	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	C 18:3	Sat: Unsat ratio
non (control)	0.4	30	0	0.5	23.6	45.2	0.3	1:2.23
Bacterial protease	0.5	29.6	0	0.4	24	45.1	0.2	1:2.27
Savinase	0.3	28.4	0.3	1.4	24.5	44.9	0.2	1:2.28
Papain	0.5	27.9	0.2	0.1	25.1	46.1	0.1	1:2.48
Termamyl	0.7	27.2	0.8	2.4	23.2	45.6	0.1	1:2.11
Savinase : Bacterial protease (1:1)	0.2	32.1	0.1	0.2	23	44.2	0.2	1:2.34
Savinase : Papain (1:1)	0.4	30.1	0.3	0.3	25	43.6	0.3	1:2.11
Savinase : Termamyl (1:1)	0.3	30	0	0.9	24.3	44.2	0.3	1:2.21
Savinase : Cellulase (1:1)	0.5	29.9	0	0.5	24.3	44.6	0.2	1:2.21
Savinase :Pectinase (1:1)	0.3	32.2	0.2	0.1	23	44	0.2	1:2.01
Savinase:Cellulase:Pectinase (0.66 :0.66 : 0.66)	0.3	29.9	0	0.2	24.2	45.2	0.2	1:2.28
Savinase:Bacterial protease: Pectinase (0.66 : 0.66 : 0.66)	0.3	30.2	0.2	0	24.6	44.4	0.3	1:2.25

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