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Biodegradation of agro-industrial orange waste under solid state fermentation and natural environmental conditions

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ABSTRACT
Evaluation of the possibility of the re-use of agro-industrial orange peel and pulp wastes under solid state fermentation and natural environmental condition as a source of enzymes production [α & β amylase, cellulase, pectinase(s), lipase(s), esterase(s) and peroxidase(s)] the physiological enzymes of lysis and total protein. Different microorganisms such as fungi, bacteria and yeast which were charged of waste analyse and have ability to produce previous enzymes and protein. These microorganisms were isolated from the fermented waste and preliminarily identified to test each one of them for their enzymes production and also to test them for inducing the fermentation process under the natural conditions, or by using one of the present enzyme producer strain. The protein and physiological enzymes production were electro-phoretically patterned in different bands. And finally, the possibility to use the fermented waste as a bio-fertilizer was done and it stimulated the growth of wheat plant using water culture, especially after the toxicity of the fermented waste was investigated.

KEYWORDS: Solid state, peel & pulp waste, enzymes, protein, bio-fertilizer, bacteria, fungi, yeast.

INTRODUCTION

Solid State Fermentation methods are widely used for the industrial production of microbial enzymes and metabolites. A variety of solid substrates have been identified for use in the production of several enzymes such as cellulase, α amylase and pectinase (Toyama 1963) and lipase (Yamada 1977). The citrus processing residues are rich in both soluble and insoluble carbohydrates (Kesterson & Braddok 1976) which makes them an attractive potential feedstock for biological conversion to value added products. The traditional re-use of orange pulp and peel waste ential a high cost, impractical for Egyptian citrus industries, and therefore these industries have been accumulating peel and pulp waste in the soil, causing serious invironmental problems.

Peel, pulp and membranes from oranges and other related citrus fruits are highly susceptible to hydrolysis by mixtures of cellulolytic and pectinolytic enzymes (Grohmann & Baldwin 1992). Mixtures of cellulase and pectinase enzymes are necessary for the complete conversion of all carbohydrates to monomeric sugars (Grohmann *et al.* 1994) special attention was paid to carbohydrate enzymes produced by fungi which are industrially important in the clarification and extraction of fruit juices and in degamming of natural fibers in the textile industry. The enzyme pectinase was of particular interest because it is involved in the phytopathogenic process caused by fungi (Fogarty & Kelly 1983). The re-use of agro-industrial orange waste as organic fertilizers seems to be a low cost technology for the recycling of the nutrients contained in this waste. Therefore the objective of this work is not only to overcome the problems of the orange peel and pulp waste accumulation in the environment by converting them under solid state fermentation into useful products such as the production of carbohydrate lyses enzymes, (α & β) amylase, cellulase, pectinase(s) and lipase(s), physiological enzymes, esterase(s), peroxidase(s) in band profile and the total protein, but also to minimize the time of fermentation. In addition to use the fermented waste as a bio-fertilizer.

MATERIALS AND METHODS

Raw materials and preparation of precursor culture for solid state fermentation: Fresh orange peel and pulp waste was collected separately from Kaha factory. The so called "starter

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culture" was prepared as follows: 25 g of fresh orange waste was incubated in two types of bottles (glass and polyethene) with capacities of 300, 500, and 1500 ml. Incubation continued at room temperature for 15-20 days under natural environmental conditions. At the end of the incubation period, the orange waste was a completely solubilized mixture, which was used as an inoculum "precursor" for fresh new agro-industrial orange waste to minimize the time of fermentation. *Triticum vulgare* C.V. "Giza 164" was obtained from the Crop Research Institute, Ministry of Agriculture, Egypt, Cairo and was used as a bioassay seedling growth.

Growth media: Nutrient agar, Sabouraud and yeast-extract agar media were used for the isolation and purification of bacteria, mould and yeast respectively.

Identification of micro-floral isolates: The bacterial isolates were preliminarily identified using Bergey's Manual (Williams *et al*. 1989), while Samson (1979) were used for fungal identification, various yeast strains were isolated but could not be identified. All the pre-mentioned micro-floral isolates were isolated from the agro-industrial fermented waste of orange peel and pulp.

Extraction of lysis enzymes: The enzymes examined in the present study were extracted from fermented orange waste and their identified micro-floral organisms according to the procedure of Kar and Mishra, (1976).

Lysis enzyme assays: Quantitative α, β amylase and cellulase activities were determined as according to Malik & Singh (1980) while qualitative and quantitative activities of pectinase(s) and lipase(s) were carried out using the clearing-zone technique described by Ammar *et al*. (1995, 1994) and Elwan *et al*. 1977) respectively.

Isozymes and protein electrophoresis: One gram from each of fermented orange peel and pulp waste samples were pelleted by centrifugation at 13000 rpm. for 10 min. The protein extracted using protein extraction buffer contains 5 ml tris/borate pH 8.9 containing 5% SDS "sodium deodocyle sulfate" and 5 ml cold distilled. H_2O for isozymes and 2 h. shaking according to Hussein & Stegemann (1978). Polyacralamide standard gel (5%) was performed on vertical slabs using the biored gel electrophoresis system. The run was performed at 300 v for 2 h.

Isozymes and protein visualization: Coomassi brilliant blue R 250 was used in protein staining, where 5 ml of 1% stain were added to 200 ml of a mixture of 60 g trichloroacetic acid, 800 ml water, 200 ml ethanol and 70 ml acetic acid. The gels were stained over night. The destaining was composed of 300 ml methanol, 700 ml water, and 50 ml acetic acid. For esterase(s) and peroxidase(s) isozyme visualization was achieved following the methods Scandalios (1964) and Larsen & Beuson (1970), respectively.

Orange peel and pulp fermented applications on *Triticum vulgare* seedling: *Triticum vulgare* seeds were treated with mixture of soluble fermented waste (peel or pulp) and water (1:10 ml v/v) for 24 h. untreated and treated seeds were germinated in 15 cm petri dishes (20 seeds per dish) then transferred into growth chamber under 12 h. light/day at 30±2°C and left to grow for 3 weeks with three replicates for each treatment. After that, observations were made of shoot and root length, number of leaves, fresh and dry weight.

Statistical analysis: All results were submitted to variance analysis (ANOVA), the significant differences were measured at $p< 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the different micro-floral strains which were isolated and preliminarily identified from the fermented orange peel and pulp waste. Two different fungal strains, *Aspergillus flavus* and *Nigrospora* sp. were found in orange pulp but were absent from the fermented orange peel waste. For the isolated yeast, there were three different isolates from the peel and pulp waste, Ashbell *et al*. (1987) showed that the orange waste often contain large numbers of yeasts. Two

bacterial strains were found in the fermented orange peel waste (rod and cocci, gram -ve and non spore former), and streptobacilli strain (gram +ve and non spore former) were isolated from the fermented orange pulp waste but were absent from the fermented peel waste (Table 1).

Table 1: Preliminary identification of the isolated microflora from the fermented orange peel and pulp wastes. The three yeast isolates were not identified but they were different according to their difference in their enzymatic production.

The isolated strains from orange peel	The isolated strains from orange pulp
Fungi: *Aspergillus nidulans & fumigatus*	**Fungi**: *Aspergillus nidulans*, *fumigatus* & *flavus*, *Nigrospora* sp
Yeasts: Y_1 isolate	**Yeasts:** Y_2 isolate & Y_3 isolate
Bacteria: Strain No. 1: Streptobacilli, gram +ve, terminal spore former. Strain No. 2: Streptobacilli, gram +ve, central spore former. Strain No. 3: Cocci, gram -ve, non spore former. Strain No. 4: Rod, gram -ve, non spore former.	**Bacteria:** Strain No. 1: Streptobacilli, gram +ve, terminal spore former. Strain No. 2: Streptobacilli, gram +ve, central spore former. Strain No. 3: Streptobacilli, gram +ve, non spore former.

The results in tables 2 & 3 include a comparative study between carbohydrate and lipase enzymes that had been extracted from the fermented peeled and pulp and micro-floral organisms. In the peeled fermented waste, *A. nidulans* was the best microorganism for α-amylase production (103.83 μg/ml) while yeast strain 2 found in the pulp was the best for α-amylase production (631.00 μg/ml), representing six times the value of that produced by *A. nidulans* in the peel. For the production of β amylase in the micro-floral enzymes from the peel, yeast strain 1 produced the highest value (4566.00 μg/ml), while in the pulp yeast strain 3 showed the highest value (9695.70 μg/ml) which is twice the value produced in the peeled waste. In addition yeast strain 1 showed the highest value of cellulase production (221.76 μg/ml), while yeast strain 2 in the pulp showed the best yield (201.60 μg/ml). It can be deduced that we obtained two unusual strains of cellulytic activity, because yeasts in general do not show an affinity towards cellulase production, while microflora in the peel showed slight activity of cellulase production and its corresponding in the pulp. For the last carbohydrate enzyme pectinase(s), *A. fumigatus* produced the highest quantity of micro-floral pectinase(s) enzyme (1258.90 unit/ml) in the peel, which is in agreement Kester & Visser (1990) which showed that *Aspergillus* strains were synthesizing a mixture of several enzymes. *A. fumigatus* strain used in our experiment probably excreted several additional enzymes, while *Nigrospora* sp. in the pulp showed the highest activity (28183.80 unit/ml) in pectinase(s). Further more, *Nigrospora* sp. recorded high activity (1000 unit/ml) in lipase production, while the highest activity in the peel was recorded by bacterial strain 1 (Tables 2 & 3). In addition, there is a high productivity of cellulase in contrast with the pectinase(s) activity and do not produce appreciable amounts of pectinase which shows agreement with Grohmann & Baldwin (1992). Using enzymatic hydrolysis much better than the acidic hydrolysis of polysaccharride tissue, this gives agreement with Grohmann *et al*. (1995) which showed that enzymatic hydrolysis is more sensitive than acidic hydrolysis of polysaccharide in plant tissues because the hydronium is a less selective catalytic agent for this reaction. Numerous glycosidic bonds can be broken at similar rates (Timell 1964) with the exception of cellulose, which is relatively resistant to acid-catalyzed hydrolysis. This is due to its insolubility and crystallinity (Philipp *et al*. 1979). In addition, pectin, in which glycosidic bonds between galacturonic acid units appear to be more resistant due to a combination of inductive and conformation effects (de Vries 1988).

When using cellulase and pectinase(s) enzymes approximately 90% of the waste is solubilised (Grohmann *et al*. 1995). Higher solubilization of the orange waste by enzymatic rather than by acid treatment is mainly caused by enzymatic depolymerization and

solubilization of cellulose. Hydrolysis of untreated substrate is a very efficient treatment for the solubilization of total wastes. Peel is highly susceptible to enzymatic hydrolysis. Penetration by enzymes is aided by the disintegration of tissues and soluble forms of polysaccharides are generally easier to hydrolyse than in soluble, particulate forms.

Table 2: A comparative study for the determination of peeled micro-floral enzymes

Micro-floral strain tested	A-amylase µg/ml	B-amylase µg/ml	Cellulase µg/ml	Pectinase(s) unit/ml	Lipase unit/ml
A. nidulans	103.83	2462.40	185.47	588.80	800.10
A. fumigatus	72.58	2846.50	211.68	1258.90	690.100
Y_1 sp.	10.48	4566.00	221.76	not detected	585.00
Strain (1)	7.66	30.80	14.11	39.80	1000.00
Strain (2)	18.14	100.20	16.12	25.11	191.30
Strain (3)	11.29	100.30	10.08	19.05	182.60
Strain (4)	10.08	46.20	18.14	not detected	68.50

F (for enzymes) =1277.87, $p< 0.05$. F (for strains) = 600.89, $p< 0.05$. Value are the means of three replicate enzymes.

Table 3: A comparative study for the determination of pulp micro-floral enzymes

Micro-floral strain tested	α-amylase µg/ml	B-amylase µg/ml	Cellulase µg/ml	Pectinase(s) unit/ml	Lipase unit/ml
1- *A. nidulans*	332.64	1744.20	139.10	3.98	80.00
2- *A. fumigatus*	106.44	3898.80	171.36	25.11	90.00
3- *A. flavus*	436.75	4719.60	153.22	2.23	200.00
4- *Nigrospora* sp.	4.032	205.20	72.58	28183.80	1000.00
5- Y_2 sp.	631.00	179.50	201.60	63.09	191.30
6- Y_3 sp.	82.66	9695.70	38.28	not detected	100.00
7- Strain (1)	8.26	35.90	9.88	not detected	100.00
8- Strain (2)	9.88	102.60	14.11	not detected	182.60
9- Strain (3)	11.89	153.90	23.99	not detected	200.00

F (for enzymes) =148402.02, , $p< 0.05$. F (for strains) =141709.90, $p< 0.05$. Value are the means of three replicate enzymes

The results recorded in Table 4 show that the enzymes produced from the fermented orange peel waste corresponds to the type and capacity of the bottles were used. The maximal production obtained from each of the following enzymes were: α amylase 219.744 µg/ml, β amylase 1641.60 µg/ml, cellulase 84.67 µg/ml, pectinase(s) 1584.89 unit/ml (carbohydrate enzymes) and lipase(s) 1233.00 unit/ml, while the corresponding bottles were used green plastic (1500 ml), transparent plastic (300 ml), transparent glass (500 ml), green glass (300 ml) and transparent glass (500 ml). In Table 5 showed the highest value from each of: α amylase 94.652 µg/ml, β amylase 1898.10 µg/ml, and cellulase 78.62 µg/ml and the transparent plastic (300 ml) bottle was the best bottle for their production while for production of pectinase(s) 1548.90 unit/ml, green glass (300 ml) had been used and for lipase(s) 690.10 unit/ml, transparent plastic (500 ml) was used. From tables 4 and 5, it can be deduced that for the best production of α amylase, cellulase, pectinase(s) and lipase(s), the orange peel was used, in contrast to the production of the highest value for β amylase, where orange pulp was used.

Results obtained for the production of enzymes as a parameter for solid state fermentation from a substrate of orange peel and pulp demonstrated clearly the impact of the process parameter on the gross yield of enzymes as well as the independent nature in influencing the organisms ability to synthesize these enzymes. In general a reduction in the amount of micro-floral enzymes production in the fermented waste than their formation in their specific media, it might be due to competition between the micro-foloral and the same substrate and impaired oxygen transfer (Sandhya & Lonsane 1994). A lower moisture content also resulted in a decline in enzyme yield. This may result from sub-optimal growth, reduced substrate swelling and high water tension during low moisture (Lonsane *et al*. 1985).

The particle size (specific surface area) is a critical factor in solid state fermented. A similar trend was reported for most of the isolated enzymes gluco-amylase from wheat bran (Pandey 1991) and for cellulase production with coir pith of small particle size (Muniswaran & Charyulu 1994). With smaller particles the surface area for growth is greater but the inter-particle porosity is less, whereas with large size the porosity is greater but the saturated surface area is less. These two opposing factors (a decrease in surface area and increase in porosity) probably interact to determine the value corresponding to optimum growth and enzyme production (Muniswaran & Charyulu 1994).

The present investigation was carried out to increase the micro-flora of orange peel and pulp waste and enhance fastening its natural biodegradation for incubation under natural environmental conditions. On using the mentioned precursor it is noted that the apparent decay was always higher when using a plastic bottles as compared to glass bottles, taking only 5 days. This is probably due to the nature and capacity of the bottles themselves, which were made of polyethene which can retain the heat temperature and allow the passage of O_2 which stimulates the growth of the micro-floral strains and so minimizes the time of solubilization and liquefaction. As far as advantages of having a higher solubilization this must be related to the microorganisms found in the subsequent fermented. By employing fungi capable of utilizing quite complex compound, also yeasts pushed depolymerization taking place (Vacarino *et al*. 1989).

Table 4: A comparative study for the determination of the peeled fermented enzymes

Bottle capacity Type & volume (ml)	α-amylase µg/ml	B-amylase µg/ml	Cellulase µg/ml	Pectinase(s) unit/ml	Lipase unit/ml
1- Green glass 300ml	102.816	1179.90	54.43	1584.89	400.00
2- Transperant 300ml (plastic)	145.152	1641.60	80.64	891.20	1166.00
3- Transperant 500ml (glass)	107.654	1385.10	84.67	not detected	1233.00
4- Transperant 500ml (plastic)	42.336	1026.00	52.42	6.30	1100.00
5- Green 1500ml (plastic)	219.744	923.40	76.61	not detected	1000.00

F (for enzymes) = 2694.87, $p< 0.05$. F (for bottles) = 216.72, $p< 0.05$. Value are the means of three replicates

Table 5: A comparative study for the determination of the pulp fermented enzymes

Bottle capacity Type & volume (ml)	α-amylase µg/ml	B-amylase µg/ml	Cellulase µg/ml	Pectinase(s) unit/ml	Lipase unit/ml
1- Green glass 300ml	15.52	513.00	46.37	1548.90	182.60
2- Transparent plastic 300ml	94.752	1898.10	78.62	6.00	300.00
3- Transparent glass 500ml	36.88	1026.00	44.35	588.80	312.50
4- Transparent plastic 500ml	60.48	1077.30	48.38	63.09	690.10
5- Green plastic 1500ml	80.64	820.80	34.27	6309.50	312.00

F (for enzymes) = 1645.66, $p< 0.05$. F (for bottles) = 731.23, $p< 0.05$. Value are the means of three replicates

Peroxidase isozyme: The banding patterns of peroxidase isozymes as illustrated in Fig. 1A & B, represents three bands only. Band No. 1 was found in all types of samples for both pulp and peeled micro-floral peroxidase isozymes with different density and intensity, while band No. 2 was observed in the pulp micro-floral isozyme only (except sample No. 3) with the same density and intensity. The last band (No. 3) was found for the pulp samples No. 1 and 3 only, while in peeled samples it was found for sample No. 8 only and with a higher density (concentration). Comparison indicated partial effectiveness of peroxidase isozymes in the identification the total micro-flora which is responsible for the biodegradation of both orange pulp and peel waste in different types and capacities of bottles and as a biochemical genetic marker.

Esterase isozyme: The banding patterns of esterase isozymes were studied in 10 samples of pulp and peel agro-industrial orange wastes. In Fig. 2 A & B, zymogram analysis by polyacrylamide gel electrophoresis for esterase isozymes showed three distinct zymogram patterns which

provided good markers for identification and characterization and also elucidated reliable biochemical markers. Band No. 1 was found in sample No. 9 only for the peeled waste, while band No. 2 is the most variable band and sample No. 3 shows the highest density and intensity. Samples 1, 2 and 3 for the pulp have the highest density and intensity, especially sample No. 3 for the pulp, while at the same time the third band was present in sample No.1 for the pulp waste only with very high density.

The result as shown in Fig. 3A & B represents six different bands over all the samples. The total micro floral protein of the pulp (1---5) and peeled (6---10) fermented wastes are shown in Fig 3 (A & B) in SDS protein electrophoresis with a molecular weight of 200, 170, 100, 70, 50 and 37 K.D. a band of 200 KD. was present in all samples except No. 4 with different intensities according to the key below. The band of 170 KD was also present in all 10 samples (except No. 9) with different intensities, while the band of 100 KD was present in samples No. 5 & 10 only with the same intensities. The band of 70 KD was present in samples No. 4 & 5 only "pulp" micro-flora, the band of 50 KD was present in all samples except sample No. 4, 5 and 9 with different intensities, but the last band of 37 KD was present in samples No. 1, 6, 8 and 10 with different intensities.

From these results it is evident that the protein electrophoretic bands could be considered as a useful tool for the identification and characterization of the total micro-floral protein that are responsible for the biodegradation for the pulp and peel agro-industrial waste and according to the different bottles capacities.

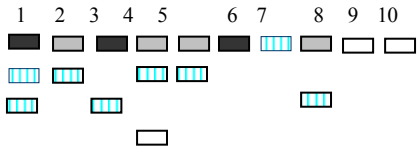

Fig. 1: Banding pattern of peroxidase of pulp 1-5 and peeled 6-10 orange fermented waste

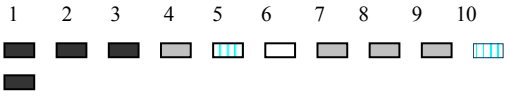

Fig. 2: Banding pattern of esterase of pulp 1-5 and peeled 6-10 orange fermented waste

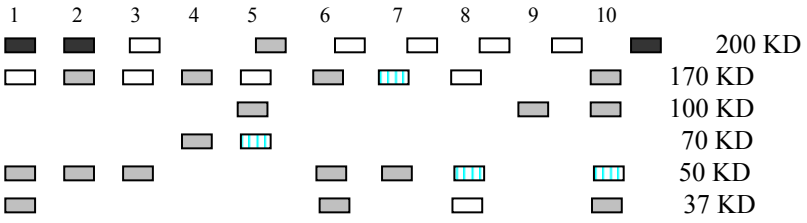

Fig. 3: Banding pattern of total protein of pulp 1-5 and peeled 6-10 orange fermented waste in 200, 170, 100, 70, 50 and 37 KD.

very high density high density moderate density slight density

Starch consists of amylose and amylo pectin, α-amylase hydrolyses the linear amylose chain producing a mixture of maltose and glucose, while β-amylase attacks amylose to produce successive units of maltose. As the seeds germinate, the process of mobilization of food reserves starts. There are some soluble reserves in the seeds which are utilized during the early phase of germination. However, for the later stages of growth, conversion of insoluble reserves to soluble forms is needed to supply the required nutrition for the embryo growth. Starch is one of the main food reserves in whose mobilization α-amylase plays the main role. Specific activity of α-amylase activity increased with seedling growth (Jain & Khanna 1986). β-amylase activity is

quite high in our treatment (Table 6 for peeld and pulp). The results in Table 6 showed a great stimulating effect on the seedling growth of *Triticum vulgares* seeds.

The maximum growth parameters on using transparent glass bottles for peeled fermentation (500 ml) and green plastic bottles (1500 ml) respectively when compare with the control (water treatment), while the maximum growth parameters on using the fermented pulp in transparent plastic bottles (300 & 500 ml) respectively (Table 6). These can be referred to the activity of α and β-amylase enzymes (Jain & Khanna 1986).

In conclusion, from the previous data in the present investigation, it can be concluded that the production of the best amount of the corresponding enzymes, α and β amylases and pectinase(s) we must inoculated the orange pulp waste with the corresponding strains, Yeast strain 2, Yeast strain 3 and *Nigrospora* sp. respectively. However, the maximal production of the cellulase and lipase enzymes we use the orange peel waste and inoculated with yeast strain 1 to produce cellulase enzyme, in contrast for lipase(s) production we use the fermented peel waste. This conclusion can be applied on a large scale especially all types of the fermented wastes are free from any type of toxins, we deduced this notice experimentally. Further more, the solubilized fermented orange peel and pulp waste can be used as a bio-fertilizer which enhance the seedling growth of wheat.

Table (6): Effect of fermented peeled and pulp on seedling growth parameters of *Triticum vulgare*.

Treatment	Fresh wt. (gm)	Dry wt. (gm)	No. of leaves/ plant	Shoot length (cm)	Root length (cm)
Untreated (control)	3.0	0.34	5	12.1	4.3
Peeled fermented in green glass bottle (300 ml).	3.0	0.83	6	16.8	10.0
Peeled fermented in transparent plastic bottle (300 ml)	2.1	0.50	6	14.6	6.80
Peeled fermented in transparent glass bottle (500 ml)	3.5	0.95	6	18.5	17.0
Peeled fermented in transparent plastic bottle (500 ml)	2.9	0.80	6	16.1	8.8
Peeled fermented in green plastic bottle (1500 ml)	3.2	0.84	6	17.6	11.8
Pulp fermented in green glass bottle (300 ml).	1.91	0.30	6	6.6	1.3
Pulp fermented in transparent plastic bottle (300 ml).	5.10	1.14	6	19.5	8.0
Pulp fermented in transparent glass bottle (500 ml).	2.68	0.93	5	16.8	8.0
Pulp fermented in transparent plastic bottle (500 ml).	3.86	1.10	5	19.0	16.0
Pulp fermented in green plastic bottle (1500 ml).	1.95	0.61	5	13.3	9.6

F (for parameters) = 227.61, $p < 0.05$. F (for treatment) = 9.71, $p < 0.05$.
Value are the means of three replicate for the treatments (control, treated) and the parameters

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الملخص العربى

التحليل الحيوى لمخلفات المصانع الزراعية من قشر وأب البرتقال تحت ظروف تخمر الحالة الصلبة والظروف البيئية الطبيعية

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فى محاولة للتقييم والإستفادة من إعادة إستخدام مخلفات المصانع من قشر وأب البرتقال تحت ظـروف تخمـر الحالـة الصلبه والظروف البيئية الطبيعية .. فقد تم إنتاج بعض الإنزيمات المحللة (ألفا – بيتا أميليز، سيليوليز، بكتينيز، ليبيز). كما تم عزل وتعريف بعض الميكروبات تعريفاً مبدئياً من بكتيريا وفطريات وخمائر والمسئوله عن تحلل هذه المخلفات وإمكانية إنتاجها لهذه الإنزيمات. أيضاً تم تحليل بعض الإنزيمات الفسيولوجية من بيرأوكسيديز وإسـتيريز والبـروتين الكلى فى المخلفات الذائبة المتخمرة تحليلاً كهربائياً للتعرف على التركيب الجزيئى للمركبات. وفى محاولة لإسـتخدام المخلفات الذائية المتخمرة كسماد حيوى فقد وجد أنها تساعد على تنشيط معدل النمو فى نبات القمح فى المزرعة المائية خاصاً بعد التأكد من خلوها من أنواع السميات.